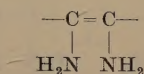


Zur Kenntnis der Endiamine

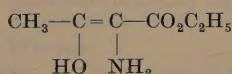
Von HANS VON EULER, HANS HASSELQUIST und HANS WÄHLSTAM

In ihrer kleinen Monographie haben EULER und HASSELQUIST (1) als einen Vertreter der durch die Gruppe



gekennzeichneten, von ihnen „Endiamine“ benannten Stoffe, das von MICHEEL (2) dargestellte 3,4-Diamino-tetron angeführt. MICHEEL hatte dieses Endiamin aus Tetronsäure gewonnen, indem er das Bis-phenylhydrazon der Dehydro-oxytetronsäure in neutraler Lösung katalytisch mit Pd hydrierte.

Er hatte die von ihm betonte starke Reduktionswirkung des genannten Endiamins sowie des ebenfalls von ihm dargestellten 5-Glykol-derivates nicht mit Tillmans-Reagens sondern mit Jod sowie mit Silbernitrat in neutraler Lösung konstatiert. Wie MICHEEL angibt, sind die beiden Endiamine schwächere Reduktionsmittel als die Amino-Reduktone (Enaminole), z. B. als der von MARTIUS und KNOOP (3) dargestellte α -Amino- β -ketobuttersäure-ester bzw. dessen Enolform



oder als das 3-Amino-4-hydroxy-derivat des Tetrons.

Vor einiger Zeit haben wir (4) die Reduktionswirkung der Reduktone anlässlich von Versuchen über den Einfluss der Acidität auf die Aktivität der Reduktone kurz besprochen. Beim aci-Endiol Ascorbinsäure hatten sich die folgenden Entfärbungszeiten ergeben, mit einem Maximum von 33 Sekunden bei pH = 9,6.

Ascorbinsäure, pH	5	7	9,6	10,5	11,5
Sekunden	15	29	33	29	2,5

Wie früher hatten wir darauf hingewiesen, dass die Geschwindigkeit dieser Reaktion auch durch die andere Komponente, nämlich das Tillmans-Reagens, bedingt ist. Abgesehen hiervon ist aber für die Geschwindigkeit dieses, wie anderer analoger Reaktionen, maassgebend 1) die Lage des Endiol-Keto-Gleichgewichtes, 2) die spezifische Aktivität des Endiol-Moleküls.

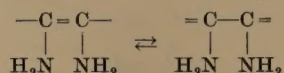
Aber auch wenn diese bekannt ist, wird noch keine endgültige Einsicht gewonnen, in welcher Weise die Struktur, die Atomabstände und die Verteilung der Ladungen im elektrischen Feld des Moleküls die Reaktionsfähigkeit der Endiolhydroxyle be-

stimmen, und inwieweit das Maximum der Reaktionszeit von der Lage eines isoelektrischen Punktes des Endiolkörpers beeinflusst wird.

Bei der erwähnten Besprechung (4) sind wir auf den Einfluss der Acidität auf Endiamine zurückgekommen. Bei einem aromatischen Endiamin, dem *o*-Phenylendiamin fanden wir, dass die Geschwindigkeit der Reaktion mit Jod mit zunehmendem pH wie folgt *zunimmt*:

<i>o</i> -Phenylendiamin, pH	1,1	1,6	5,1
Sekunden	1920	360	140

Nun bilden aromatische Endiamine eine besondere Endiamin-Gruppe, was auch der Sonderstellung der aromatischen Endiole vom Typus des Brenzcatechins und seiner Derivate (Adrenalin u. a.) entspricht. In beiden Fällen muss man die Oscillation der Doppelbindung in Betracht ziehen:



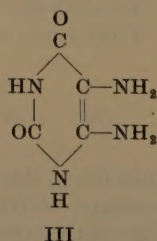
Die Geschwindigkeit der Reaktion, mit welcher Dichlorphenol-indophenol (Tillmans-Reagens, TR) von *o*-Phenylendiamin entfärbt (nicht reduziert) wird, *sinkt* mit steigendem pH.

Bezüglich des 3,4-Diamino-tetrons von MICHEEL wurde schon eingangs erwähnt, dass dieses Endiamin vom genannten Autor nicht an Tillmans-Reagens (TR, Dichlorphenol-indophenol) geprüft wurde. Es erschien uns wünschenswert, in dieser Hinsicht eine Ergänzung vorzunehmen. Dabei hat sich folgendes gezeigt:

Das Diamino-Tetron lässt sich in neutraler oder saurer Lösung durch Tillmans-Reagens wegen Rotfärbung nicht titrieren. In schwach alkalischer Lösung entfärbt es TR nur langsam. Verbrauch: 0,82 Mol TR per Mol Tetron.

Dagegen verbraucht 1 Mol des Diamino-tetrons 1,06 Mol J₂, was dem Ergebnis von MICHEEL entspricht.

Man kennt nun auch andere Diamine, die man als *aci*-Diamine bezeichnen könnte. Unter diesen scheint am besten untersucht zu sein das 3,4-Diamino-uracil, das von BREDERECK (5) bearbeitet wurde. Man formuliert diese einwertig funktionierende, beständige Base folgendermaassen (III):

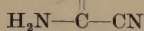
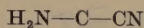


BREDERECK hat die Konstitution der aus dem Diamino-uracil gewonnenen Acetate, und in einer weiteren Arbeit (6) deren Umsetzungen zu Purin-derivaten, besonders die Methylierungen, untersucht. W. TRAUBE (7) setzte das Diamino-uracil mit Chlorkohlensäure zu einem Urethan und über dasselbe zu Harnsäure um.

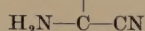
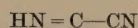
Bemerkenswert ist in biochemischer Hinsicht die Bildung von *Pteridinen* nach PFLEIDERER (8).

In neuester Zeit hat BREDERECK (9, 10) die Konstitution der *tetrameren Blausäure* und ihrer *Acyl-Derivate* sowohl chemischen wie spektroskopischen (auch im Ultrarot) Untersuchungen unterworfen und hat eine neue Methode zur Darstellung dieses interessanten Materiales angegeben.

Schon vor mehr als 80 Jahren hatte O. LANGE (11) polymerisierte Blausäure in fester, krystallisierter Form erhalten, die 1923 von BEDEL als tetramere Blausäure erkannt wurde. GRYSKIEWIECZ-TROCHINOWSKI (12) stellte für dieselbe zuerst die Formel I auf, welche



I



II

einem Endiamin entspricht. HINKEL (13) betrachtete auf Grund neuer Versuche die Struktur II als wahrscheinlicher. Weitere chemische Umsetzungen sowie physikalische Messungen haben dann R. L. WEBB, S. FRANK und W. C. SCHNEIDER (14) ausgeführt; sie neigen der Auffassung zu, dass der tetrameren Blausäure die Struktur I zukommt. Auch die von BREDERECK durchgeführten Umsetzungen der tetrameren Blausäure mit Aldehyden, mit Essigsäureanhydrid, mit Acetylchlorid und mit Imidoestern lassen eine Struktur I möglich erscheinen. Es frug sich nun, ob sich die tetramere Blausäure gegen Tillmans-Reagens so verhält, wie man von einem Endiamin erwarten kann.

In neutraler oder schwach saurer Lös. (pH ~ 4) wird Tillmans-Reagens *nicht* entfärbt. Bei pH ~ 10 verbrauchen 3 mg (HCN)₄ unter Stickstoff ziemlich schnell 0,037 Äquiv. TR.

Äquiv. Gew. = 81 statt für Endiamin ber. = 54. Somit TR-Verbrauch ~ 70 % des für Endiamin berechneten Wertes.

Jodtitration: In KAc-Pufferlös. verbrauchen 2,6 mg (HCN)₄ 0,0335 Äquiv. Jod, somit 70 % des für Endiamin berechneten Wertes.

Diamino-Derivate höherer Kohlenwasserstoffe haben wegen ihrer Beziehung zu *Necrosamin* (SHEAR, (15); IKAWA, (16)) auch biologisches Interesse.

Auf die Besprechung solcher Diamine (17) und ihrer Gleichgewichte, besonders des 4,5-Diamino-*n*-eicosans und entspr. *Dione*, *Dirole* und *Pseudo-Reduktone*, sowie auf das Verhalten bekannter Diamine und Amidine zu TR und J₂ kommen wir an anderer Stelle zurück.

Stockholm, Universität, Institut für organ.-chemische Forschung, Dezember 1956.

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Tryckt den 27 februari 1957

Uppsala 1957, Almqvist & Wiksells Boktryckeri AB

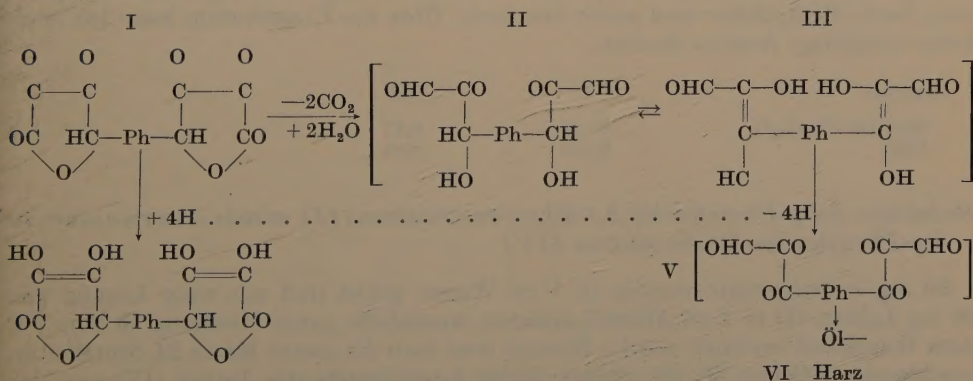
Über die Entstehung von aci-Reduktionen bei der Spaltung der 4-Aryl-2,3-diketo-butyrolaktone. II

Von HANS HASSELQUIST

In einer vorhergehenden Mitteilung (1) wurde die Bildung von aci-Endiolen beschrieben, die durch Erhitzung von 4-Aryl-2,3-diketo-butyrolakton in Wasser mit Na-Acetat als Katalysator eintritt. Bei dieser Bildung konnte eine kleine Menge des aci-Endiols Subst. 229 isoliert werden, die in kristallinischer Form bei der Spaltung von *p*-Phenyl-bis-2,3-diketo-butyrolakton (I) entsteht, das TR in saurer Lösung schnell entfärbt. Da die Eigenschaften dieser Substanz gut mit denen des 5-*p*-Phenyl-bis-3,4 dioxytetrons (IV) übereinstimmen — sie zeigen den gleichen Schmelzpunkt, 300°, ferner die gleiche Löslichkeit in verschiedenen Lösungsmitteln; die TR-Titration gab für Subst. 229 $E = 71$, für das Tetron (Subst. 231) $E = 64$ — wurde zwecks Identifizierung eine grössere Menge der Substanz 229 dargestellt. Auskristallisiert aus Wasser zeigten die beiden Stoffe geringe Ungleichheit der Kristallformen, was jedoch, späteren Ergebnissen zufolge, auf der verschiedenen Kristallisationsgeschwindigkeit beruhte.

Im Papierchromatogramm zeigten die beiden Substanzen den übereinstimmenden Wert $R_f = 0,84$. Durch Methylierung der beiden Substanzen mit Diazomethan-Ätherlösung wurden Methyläther vom gleichen Schmelzpunkt 206° erhalten; die Bestimmung eines Mischschmelzpunktes ergab keine Depression. Die Substanz 229 war somit zweifellos mit 5-*p*-phenyl-bis-3,4-dioxytetron identisch.

Bei der Erhitzung von *p*-Phenyl-bis-2,3-diketobutyrolakton in wässriger Lösung mit Na-Acetat müssen also de zuerst entstandenen Spaltprodukte II und III einen Teil des unveränderten Butyrolaktone zu Tetron reduzieren, das sich bei der Erhit-



zung in der wässrigen Na-Acetatlösung stabil erweist. Die in dieser Weise dehydrierten Spaltprodukte V und VI bilden nach Abschluss der Reaktion eine ölige Masse.

DAHN und HAUTH (2) konnten auf diese Weise zeigen, dass sich bei der Behandlung von 4-Phenyl-2,3-diketobutyrolakton mit wässriger Na-Acetatlösung gleichzeitig mit den Spaltprodukten auch 4-Phenyl-2-hydroxytetronsäure bildet.

Lakton I liess sich leicht zum Tetron IV mittels H_2S reduzieren (1) und diese Reduktion wurde ergänzt durch Reduktion mittels Ascorbinsäure, die sich leicht mit nahezu quantitativer Ausbeute durchführen lässt. DAHN und HAUTH (2) haben darauf hingewiesen, dass mit dieser neuen Methode 4-Phenyl-2-hydroxytetronsäure durch Reduktion von 4-Phenyl-2,3-diketobutyrolakton mit Ascorbinsäure oder Reduktion erhalten wird.

Experimenteller Teil

Papierchromatographischer Versuch mit Subst. 229 und 5-p-phenylen-bis-3,4-dioxytetron (Subst. 231)

Papier: Munktells OB. Lösung: *n*-Butanol-gesättigtes Wasser. Entwickler: Tillmans-Reagens (TR) gelöst in Alkohol zur Konz. 0,005-*n*. Das Chromatogramm wurde hervorgerufen solange das Papier noch feucht war, um eine ev. eintretende Oxydation während des Trocknens zu vermeiden. Die beiden Substanzen gaben übereinstimmende R_f -Werte.

Methylierung der Substanzen 229 und 231 mit Diazomethan

20 mg Subst. 229 wurden mit einem Überschuss von Diazomethan-Ätherlösung methyliert, wobei die Subst. unter lebhafter N_2 -Entwicklung in Lösung ging. Sofort nach Aufhören der Gasentwicklung wurde die Lösung bei 20° im Vacuum zur Trockne verdampft, wodurch eine gelbliche Kristallmasse und ein wenig Öl erhalten wurde. Durch vorsichtigen Zusatz von etwas Äthylacetat konnten die Kristalle leicht vom Öl getrennt werden, das leicht in Lösung ging.

Rohprodukt 7 mg vom Schmp 204°. Beim Umkrystallisieren aus wenig Äthylacetat entstanden farblose Prismen vom Schmp 206°.

In gleicher Weise wurde die Subst. 231 methyliert; ihr Methyläther ebenfalls den Schmp 206°. Der Mischschmelzpunkt der beiden Methyläther ergab keine Depression; beide Methyläther sind somit identisch. Über die Konstitution kann ich noch keine endgültige Angabe machen.

<i>Analyse</i>	C	H
Ber. für $C_{20}H_{24}O_8$	61,21	6,17
Gef.	60,80	6,07

Reduktion des p-Phenylen-bis-2,3-diketo-butyrolaktone (I) mittels Ascorbinsäure zu 5-p-Phenylen-bis-3,4-dioxytetron (IV)

50 mg Ascorbinsäure wurden in 1 ml Wasser gelöst und mit einer Lösung von 50 mg Lakton (I) in 1 ml Alkohol versetzt, worauf die ganze Mischung 10 Min. auf dem Wasserbad erwärmt wurde. Hierauf liess man die ganze Masse 24 Stunden im geschlossenen Gefäss bei 20° stehen. Dabei kristallisierte das Tetron (IV) aus, das

abfiltriert und mit wenig Wasser-Alkohol gewaschen wurde. Ausbeute 46 mg, die unter Zersetzung bei 300° schmolzen.

Die Substanz wurde noch weiterhin identifiziert durch Überführung in den Methyläther mittels Diazomethan, wobei der gleiche Methyläther erhalten wurde wie für Tetron IV.

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Tryckt den 27 februari 1957

Uppsala 1957. Almqvist & Wiksells Boktryckeri AB

Copper complexes in alkaline ethylenediamine solutions

A spectrophotometric study

By HANS VINK

With 5 figures in the text

The present work is part of an investigation on the properties of cupriethylenediamine solutions of cellulose. The solvent (shortened CED) is an aqueous solution of cupric hydroxide in en¹ and it dissolves cellulose by complex formation between copper and cellulose [1, 2]. As little is known about copper complexes in these solutions, this investigation was undertaken.

Cupric ethylenediamine complexes in neutral aqueous solutions have been extensively investigated in the past, and the structure of the complexes existing in these solutions can be considered to be definitely known [3]. There exist mono-, di- and triethylenediamine complexes with the formulae Cuen^{2+} , Cuen_2^{2+} and Cuen_3^{2+} . The first two of these have chelate structure and possess very high formation constants. In the triethylenediamine complex two of the en molecules are attached as chelating groups and form a plane square, as in the diethylenediamine compound, whereas the third is attached through only one amino group to the copper atom. The copper atom has the coordination number five in this complex and it is quite unstable.

When alkali is added to a cupric solution of en new complexes are formed. This is indicated by the profound change in the extinction curves and also by the fact that the solubility of cupric hydroxide in en solutions is increased by making the solutions more alkaline [4].

A closer analysis of the extinction curves indicates that there are at least two different hydroxy complexes that are formed with alkali. Figure 1 shows extinction curves for solutions of different alkalinity. At moderate alkalinity there is an isobestic point at about 585 m μ indicating that only one new complex is formed. However, when more alkali is added, the intersection point moves to longer wavelengths, showing that new complexes are being formed.

Figure 2 shows extinction curves for solutions of the same alkalinity but with different en concentrations. Here, a marked change in absorption is observed at first, but with increasing en concentration the curves converge to a limit, where further addition of en will be without effect. On the other hand, change of the alkalinity of the solution still affects the extinction curves. When much higher en concentrations are used the extinction curves are again changed. This is due to the formation of the Cuen_3^{2+} complex.

¹ Ethylenediamine.

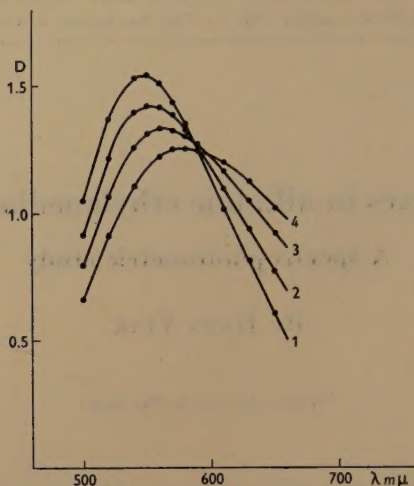


Fig. 1. Extinction curves for alkaline cupric-ethylenediamine solutions. $c_{\text{Cu}} = 0.0245 \text{ M}$, $c_{\text{en}} = 0.0514 \text{ M}$, c_{NaOH} for curves 1, 2, 3 and 4 is resp. 0, 0.167, 0.250 and 0.750 M .

From the foregoing it would appear that at high en concentrations only hydroxide ions participate in the complex formation reactions, the complexes being saturated with en. It therefore seems reasonable to postulate the following reaction

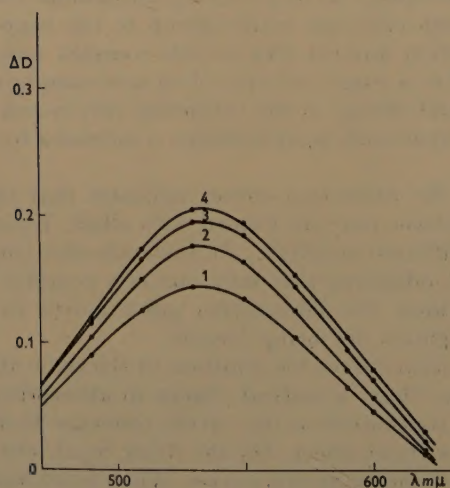
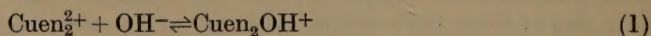


Fig. 2. Extinction difference curves for solutions with different ethylenediamine concentration. $c_{\text{Cu}} = 0.0245 \text{ M}$, $c_{\text{NaOH}} = 0.300 \text{ M}$. $c_{\text{en}} = 0.0411 \text{ M}$ in the reference solution. For curves 1, 2, 3 and 4 c_{en} is resp. 0.0741, 0.107, 0.140 and 0.173 M .

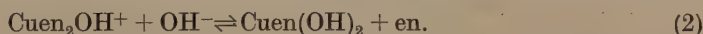
Table 1. Solubility of $\text{Cu}(\text{OH})_2$ in alkaline en-solutions.

$c_{\text{en}} = 0.1088 \text{ M.}$

c_{NaOH} mole/l	c_{Cu} mole/l	$c_{\text{en}}/c_{\text{Cu}}$
0.200	0.0628	1.73
0.250	0.0647	1.68
0.300	0.0666	1.63
0.350	0.0685	1.59
0.400	0.0713	1.53
0.500	0.0734	1.48

This complex is closely analogous to Cu en_3^{2+} , the hydroxy ion taking the place of the third en molecule. The existence of this complex has already been suggested by Jonassen *et al.* in a recent paper [5].

The existence of this complex alone cannot, however, explain the increased solubility of cupric hydroxide in alkaline en solutions. In Table 1 the solubility of cupric hydroxide in en solutions of different alkalinity is shown. It is seen that in all solutions the ratio $c_{\text{en}}/c_{\text{Cu}}$ is less than two. This fact cannot be explained by assuming that some of the copper is present as $\text{Cu}(\text{OH})_4^{2-}$ ions. The concentration of this ion in these solutions is much too low, being of the order of $0.5 \times 10^{-3} \text{ M}$ in a 0.5 M sodium hydroxide solution [6]. To explain the solubility effect and the change in the extinction curves at low en concentrations the following reaction is suggested



Here two hydroxy ions take the place of one en molecule and the plane square structure of the copper complex is conserved. Here also, one could think of the possibility of the formation of a compound analogous to $\text{Cu en}_2\text{OH}^+$ namely $\text{Cu en}(\text{OH})_3^-$, but this compound should be much more unstable because $\text{Cu en}(\text{OH})_2$ is uncharged.

Theoretical

In order to confirm these assumptions and to get quantitative information about the equilibria in the solutions, the following spectrophotometric methods were used.

When in a solution two absorbing compounds (called 1, 2) are in equilibrium with each other the optical density of the solution is given by

$$D = d \cdot \varepsilon_1 \cdot c_1 + d \cdot \varepsilon_2 \cdot c_2. \quad (3)$$

Here ε_1 and ε_2 are the molar extinction coefficients of the compounds, c_1 and c_2 their molar concentrations and d is the thickness of the absorbing layer.¹

Introducing the degree of formation of each compound $\alpha_1 = c_1/c$ and $\alpha_2 = c_2/c$, where $c = c_1 + c_2$, and the molar extinction coefficient for the solution $\varepsilon = D/d \cdot c$, we have

$$\left. \begin{aligned} \varepsilon &= \varepsilon_1 \alpha_1 + \varepsilon_2 \alpha_2, \\ \alpha_1 + \alpha_2 &= 1 \end{aligned} \right\} \quad (4)$$

¹ In this paper Beer's law is used in the form $D = 10 \log I_0/I = \varepsilon \cdot c \cdot d$, c is expressed in mole/l and d in cm.

and

$$\alpha_1 = \frac{\varepsilon - \varepsilon_2}{\varepsilon_1 - \varepsilon_2}. \quad (5)$$

Here all the ε values refer to the same wavelength. When ε values for several wavelengths are known (5) can be written more generally

$$\alpha_1 = \frac{\sum_{\lambda} |\varepsilon - \varepsilon_2|}{\sum_{\lambda} |\varepsilon_1 - \varepsilon_2|}. \quad (6)$$

When ε_1 and ε_2 are known and ε is measured, α_1 and the equilibrium constant for the reaction can easily be determined by a single extinction measurement. Usually this is not the case.

However, making use of the reaction formula, it is possible to calculate α_1 without knowing either ε_1 or ε_2 .

Suppose first that ε_2 is known. Then (6) can be written

$$\alpha_1 = f \delta, \quad (7)$$

where f is a constant $f = 1/\sum_{\lambda} |\varepsilon_1 - \varepsilon_2|$ and $\delta = \sum_{\lambda} |\varepsilon - \varepsilon_2|$. Thus δ is a measurable quantity.

It can be obtained directly by differential measurement against a solution of pure component 2, with the molar extinction coefficient ε_2 . When (7) is introduced into the equilibrium equation, an equation with two unknowns f and k is obtained. They can be determined by measuring δ for at least two different solutions.

If the reaction formula is not known, one can assume a formula. By measuring δ for a series of solutions and considering the consistency of the equations so obtained, it can often be decided whether the assumption was right or wrong.

If ε_2 is not known $\sum_{\lambda} |\varepsilon - \varepsilon_2|$ cannot be determined. On the other hand a quantity $\delta' = \sum_{\lambda} |\varepsilon - \varepsilon_2| + \text{const.}$ can be determined by measuring ε against some arbitrary standard solution. Equation (6) will then take the form

$$\alpha_1 = f \delta' + g, \quad (8)$$

where g is an unknown constant. Now three equations are necessary in order to determine the unknowns. From the viewpoint of accuracy this situation is less favourable than the first and conditions should be chosen so as to make g as small as possible.

This method can sometimes be used even when more than one reaction can take place in the solution.

Let us assume that component 2 is in equilibrium with a third compound. In this case we have instead of (4)

$$\left. \begin{aligned} \varepsilon &= \varepsilon_1 \alpha_1 + \varepsilon_2 \alpha_2 + \varepsilon_3 \alpha_3, \\ 1 &= \alpha_1 + \alpha_2 + \alpha_3, \end{aligned} \right\} \quad (9)$$

Putting

$$\alpha_2 + \alpha_3 = \alpha_2'$$

it follows that

$$\varepsilon = \varepsilon_1 \alpha_1 + \left(\varepsilon_2 \frac{\alpha_2}{\alpha_2 + \alpha_3} + \varepsilon_3 \frac{\alpha_3}{\alpha_2 + \alpha_3} \right) \alpha_2'. \quad (10)$$

The expression in the brackets represents the molar extinction coefficient of the mixture of compounds 2 and 3 and it is seen to be constant whenever

$$\frac{\alpha_3}{\alpha_2} = \text{const.} \quad (11)$$

Denoting it by ε'_2 we have the equations

$$\left. \begin{aligned} \varepsilon &= \varepsilon_1 \alpha_1 + \varepsilon'_2 \alpha'_2, \\ 1 &= \alpha_1 + \alpha'_2. \end{aligned} \right\} \quad (12)$$

These equations are the same as (4), the only difference being that the primed quantities in (12) represent mixtures of two compounds in constant proportions. Furthermore, it is quite evident that equation (4) can be generalized so that both α_1 and α_2 represent mixtures of an arbitrary number of components. It is only necessary that the components in these mixtures occur in constant proportions.

Thus it is possible, by this method, to study the equilibrium between components 1 and 2 if condition (11) can be realized and if the equilibrium between components 2 and 3 can be studied separately, so that the constant in (11) can be evaluated.

In the following these methods will be applied to the copper-en-alkali system.

It is evident from the foregoing that conditions could be chosen so that only reaction (1) takes place, the extent of reaction (2) being kept at a low value.

For reaction (1) the equilibrium equation is

$$\frac{[\text{Cuen}_2\text{OH}^+]}{[\text{Cuen}_2^{2+}][\text{OH}^-]} = k. \quad (13)$$

Here, the molar extinction coefficient of Cuen_2^{2+} , denoted as ε_2 , can be measured, since in a neutral solution with the en copper ratio slightly higher than two, almost all copper is present as the Cuen_2^{2+} ion. This follows from the magnitudes of the complexity constants as given in Table 6.

When the total copper concentration is designated by c , that of alkali by b and the degree of formation of Cuen_2OH^+ by α_1 one has

$$[\text{Cuen}_2\text{OH}^+] = c \alpha_1,$$

$$[\text{Cuen}_2^{2+}] = c(1 - \alpha_1),$$

$$[\text{OH}^-] = b - c \alpha_1.$$

When these values are inserted in (13) and α_1 is replaced with $f\delta$ from (7), (13) can be written

$$\frac{f\delta}{(1 - f\delta)(b - cf\delta)} = k. \quad (14)$$

This equation gives δ as a function of b and contains two unknown parameters f and k which can easily be determined graphically.

Equation (14) can be written in the form

$$\frac{1}{b - cf\delta} = \frac{k}{f} \cdot \frac{1}{\delta} - k. \quad (15)$$

This represents a straight line in the variables $1/(b - cf\delta)$ and $1/\delta$, the intercept being equal to $-k$ and the slope to k/f . The term $cf\delta$ is evaluated by successive approximation.

For reaction (2) we have

$$\frac{[\text{Cuen}(\text{OH})_2][\text{en}]}{[\text{Cuen}_2\text{OH}^+][\text{OH}^-]} = k_1. \quad (16)$$

Here the reactions (1) and (2) will overlap. However, from (13) it is clear that when $[\text{OH}^-]$ is kept constant the ratio

$$\frac{[\text{Cuen}_2\text{OH}^+]}{[\text{Cuen}_2^{2+}]} = k[\text{OH}^-] \quad (17)$$

will be constant. Reaction (2) can therefore be studied by the method outlined above when $[\text{OH}^-]$ is kept constant and $[\text{en}]$ is changed. Making use of the notations used above and denoting the total en concentration by c_{en} , the expressions for the concentrations in (16) take the form

$$[\text{Cuen}(\text{OH})_2] = c\alpha_1, \quad (18a)$$

$$[\text{Cuen}_2\text{OH}^+] = \frac{c(1 - \alpha_1)}{1 + \frac{1}{k[\text{OH}^-]}}, \quad (18b)$$

$$[\text{en}] = c_{\text{en}} - 2c(1 - \alpha_1) - c\alpha_1 = a + c\alpha_1, \quad (18c)$$

$$[\text{OH}^-] = b - \frac{c(1 - \alpha_1)}{1 + \frac{1}{k[\text{OH}^-]}} - 2c\alpha_1 = b' - r\alpha_1. \quad (18d)$$

Here

$$a = c_{\text{en}} - 2c,$$

$$b' = b - \frac{c}{1 + \frac{1}{k[\text{OH}^-]}},$$

$$r = c \frac{2 + k[\text{OH}^-]}{1 + k[\text{OH}^-]}.$$

When the alkali concentration b is kept constant, $[\text{OH}^-]$ will vary due to the term $r\alpha_1$. In most cases b is large in comparison with c and then the variation in $[\text{OH}^-]$ is small and can be neglected. It is also possible to compensate for the change in $r\alpha_1$ by making corresponding changes in b .

Introducing (18a), (18b) and (18c) into (16) we get

$$\frac{(a + c\alpha_1)\alpha_1}{(1 - \alpha_1)} = k_1 \cdot \frac{[\text{OH}^-]^2 \cdot k}{1 + k[\text{OH}^-]} = k_1 \cdot K. \quad (19)$$

As $[\text{OH}^-]$ may vary slightly, K in (19) may not be a constant. However, using the expression for $[\text{OH}^-]$ (18d) it can easily be evaluated by successive approximation.

In this case it is not possible to prepare a solution with its molar extinction coefficient equal to ε'_2 and the expression to be used for α_1 is therefore given by (8).

Introduction of (8) into (19) gives an equation with three unknowns f , g , and k . Having determined δ' in a series of solutions, these parameters can be evaluated. This can be done analytically by the method of least squares, which, however, is rather laborious. There are also several graphical methods that can be used [7]. The following one was found to be simplest, although it is not very accurate.

When (19) is solved for α_1 we get

$$\alpha_1 = -\frac{a + Kk_1}{2c} + \sqrt{\left(\frac{a + Kk_1}{2c}\right)^2 + \frac{Kk_1}{c}}. \quad (20)$$

With this equation, sets of α_1 values can be calculated for different k_1 values. The corresponding α_1 and δ' values will according to (8) give a straight line

$$\alpha_1 = f\delta' + g.$$

A value for k_1 is then obtained by choosing the best straight line.

Experimental

Reagents

The copper salt used was cupric nitrate and this, as well as the sodium nitrate used for the salt medium, were from "Baker's Analyzed" chemical reagents and they were used without further purification. From the copper nitrate a standard solution was prepared and its copper content was determined electrolytically.

The ethylenediamine used was from "Eastman Organic Chemicals". It was redistilled twice and from it a standard water solution was prepared. The en content of this solution was determined by potentiometric titration.

The alkali used was in the form of carbon dioxide free standardized solutions of sodium hydroxide in water.

All the water used was freed from carbon dioxide by boiling.

In one experiment, a series of measurements was made with solutions of the same copper concentration but with varying concentrations of either en or sodium hydroxide. These solutions were prepared in the following way. Two solutions were prepared by pipetting the reagents from the standard solutions into measuring flasks and diluting. These solutions had the same copper concentration but different concentrations of the reagent to be varied. A solution with an intermediate concentration of the latter reagent was then obtained by mixing these two solutions in certain definite proportions. In this way errors due to differences in copper concentration were minimized.

Spectrophotometric measurements

The instruments used were Beckman model B and DU spectrophotometers. The model DU spectrophotometer was provided with a thermostat and the temperature of the samples could be

maintained constant to within $\pm 0.1^\circ\text{C}$. All measurements with this instrument were carried out at 25°C .

In the calculations only extinction differences enter. These were obtained directly by differential measurement using one of the solutions as reference. This gives the best accuracy as in photoelectric spectrophotometry the absolute error in extinction increases with increasing extinction values [8].

The measuring cells were calibrated and corrections applied to the extinction values obtained. Careful calibration of cells was especially important since the overall absorbance of the solutions was rather high and the errors due to differences in cell thickness could therefore be considerable.

The cells used in most measurements were 1-cm Corex glass cells. In the model B spectrophotometer 3-cm glass cells were used.

Solubility measurements

The solubility of cupric hydroxide in alkaline en solutions was determined in the following way. A solution with given alkali and en concentration was prepared. Copper nitrate was added in slight excess, the cupric hydroxide formed was separated by centrifugation and the copper concentration in solution was determined electrolytically.

Results and discussion

In the case of reaction (1) a solution with its molar extinction coefficient equal to ε_2 could be prepared. Therefore it was possible to determine $\varepsilon - \varepsilon_2$ directly by differential measurements against that solution. The most favourable region for measuring the extinction differences was found to be near the wavelength $690\text{ m}\mu$ as there the extinction difference has a maximum and errors due to the presence of other complexes are small. Measurements were made at the wavelengths 670 , 690 and $710\text{ m}\mu$ and δ was formed in accordance with (7) as a sum over these wavelengths.

In Table 2 primary values for a series of measurements are given. In Figure 3 the graphical representation of equation (15) is shown for different series of measurements.

In the case of reaction (2) the alkali concentration was kept constant and the en concentration was varied. Here differential measurements were made relative to the solution with the lowest en concentration as this solution had the lowest optical density. Then δ' was calculated, choosing the solution with the highest en concentration as reference. The molar extinction coefficient of this solution differed only a little

Table 2. Extinction measurements for reaction (1).

$c = 0.04080\text{ M Cu (NO}_3)_2$
 $c_{\text{en}} = 0.1540\text{ M}$

$d = 1.000\text{ cm}$
 $t = 25.0^\circ\text{C}$

	Solution No.				
	1	2	3	4	5
$\lambda\text{ m}\mu$					
		Optical density difference $D_n - D_1$			
670	0	0.207	0.288	0.351	0.409
690	0	0.212	0.293	0.358	0.416
710	0	0.207	0.284	0.351	0.406
Corr. for δ	0	-0.38	-0.50	-0.68	-0.75
δ	0	14.96	20.70	25.30	29.42
$b\text{ mole/l NaOH}$	0	0.08016	0.1202	0.1603	0.2004

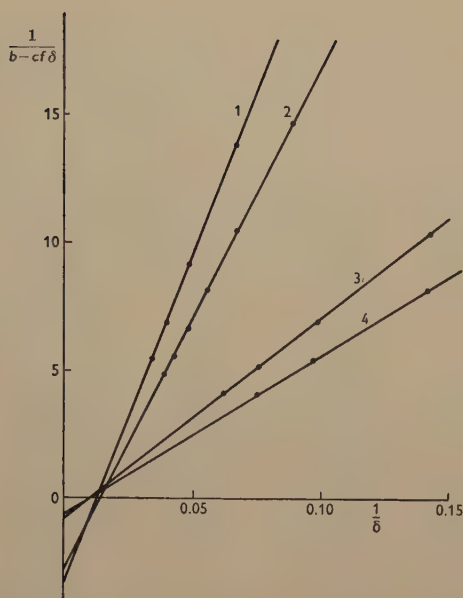


Fig. 3. Graphical representation of equation (15). c_{Cu} for lines 1, 2, 3 and 4 is resp. 0.04080, 0.1038, 0.04150 and 0.04150 M . Lines 3 and 4 represent experiments in salt media with $c_{\text{NaNO}_3} = 1.0 M$ and $1.5 M$ resp.

Table 3. Extinction measurements for reaction (2).

$c = 0.01038 M \text{ Cu}(\text{NO}_3)_2$; $d = 2.93 \text{ cm}$; $t = 23^\circ \text{C}$.

	Solution No.						
	1	2	3	4	5	6	7
$\lambda \text{ m}\mu$	Optical density difference $D_n - D_1$						
510	0	0.089	0.136	0.179	0.236	0.254	0.263
530	0	0.105	0.161	0.222	0.280	0.301	0.312
550	0	0.104	0.158	0.219	0.274	0.299	0.308
Corr. for δ'	-2.60	-0.60	-0.30	-0.14	-0.05	-0.03	0
δ'_1	26.45	18.64	13.78	8.51	3.01	0.92	0
800	0	0.064	0.105	0.153	0.200	0.220	0.228
850	0	0.063	0.105	0.151	0.196	0.216	0.225
900	0	0.057	0.095	0.137	0.179	0.198	0.206
Corr. for δ'	+0.63	-0.09	-0.15	-0.16	-0.10	-0.04	0
δ'_2	22.31	15.54	11.49	7.01	2.66	0.78	0
$\delta' = \delta'_1 + \delta'_2$	48.76	34.18	25.27	15.52	5.67	1.70	0
$c_{\text{en}} \text{ mole/l}$	0.01400	0.02286	0.03091	0.04500	0.07172	0.09060	0.1046
$b \text{ mole/l NaOH}$	0.630	0.630	0.630	0.630	0.630	0.630	0.630
δ'_1/δ'_2	1.19	1.20	1.20	1.21	1.13	1.18	

Table 4. Extinction measurements for reaction (2).

 $c = 0.0623 \text{ M Cu(NO}_3)_2$; $d = 1.000 \text{ cm}$; $t = 25.0^\circ\text{C}$.

	Solution No.					
	1	2	3	4	5	6
$\lambda \text{ m}\mu$	Optical density difference $D_n - D_1$					
510	0	0.112	0.188	0.239	0.275	0.300
530	0	0.126	0.203	0.255	0.291	0.319
550	0	0.111	0.181	0.227	0.256	0.281
Corr. for δ'	-0.41	-0.15	-0.06	-0.03	0	0
δ'_1	14.04	8.69	5.20	2.84	1.25	0
800	0	0.089	0.155	0.203	0.239	0.264
850	0	0.091	0.156	0.205	0.239	0.263
900	0	0.084	0.148	0.191	0.221	0.246
Corr. for δ'	-0.22	-0.20	-0.16	-0.11	-0.06	0
δ'_2	12.19	7.97	4.88	2.68	1.13	0
$\delta' = \delta'_1 + \delta'_2$	26.23	16.66	10.08	5.52	2.38	0
$c_{\text{en}} \text{ mole/l}$	0.1000	0.1200	0.1400	0.1600	0.1800	0.2000
$b \text{ mole/l NaOH}$	0.660	0.654	0.648	0.642	0.636	0.630
δ'_1/δ'_2	1.15	1.09	1.07	1.06	1.10	

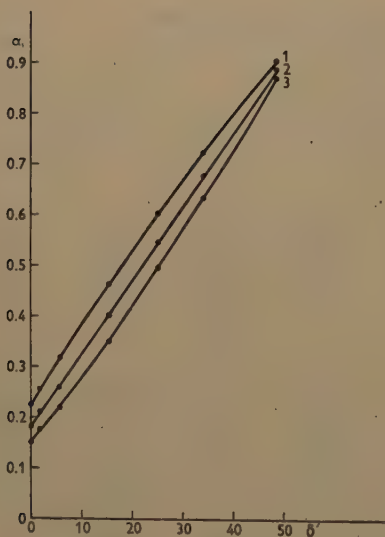


Fig. 4. Determination of k_1 according to (20) from the data in Table 3. For curves 1, 2 and 3 k_1 is resp. 0.063, 0.048 and 0.038. The k -value used is 3.0.

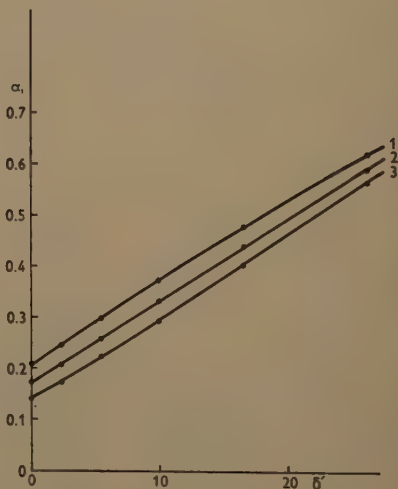


Fig. 5. Determination of k_1 according to (20) from the data in Table 4. For curves 1, 2 and 3 k_1 is resp. 0.063, 0.049 and 0.038. The k -value used is 3.0.

Table 5. Results.

$t^{\circ}\text{C}$	Reaction (1)				Reaction (2)	
	25	25	25	25	23	25
c mole/l	0.04080	0.1038	0.04150	0.04150	0.01038	0.0623
Medium			1.0 M NaNO_3	1.5 M NaNO_3	0.630 M NaOH	0.630 M NaOH
k and k_1^* resp.	3.3	2.8	0.8	0.6	0.048	0.049
f	0.0129	0.0141	0.0101	0.0097	0.0142	0.0159

* k_1 is calculated using $k = 3.0$ in (19).

from ε'_2 and therefore g in equation (8) was small. Here measurements could be made equally well near the wavelengths $530\text{ m}\mu$ and $850\text{ m}\mu$ where the extinction difference curves have maxima. In order to get a mean over a wide wavelength range the actual measurements were carried out at the wavelengths 510, 530, 550, 800, 850 and $900\text{ m}\mu$. The primary values are given in Tables 3 and 4 and in Figures 4 and 5 the graphical determination of the equilibrium constant is shown. In Table 5 the results obtained are summarized.

It remains to consider the reliability of the results obtained.

In the method applied, two main sources of systematic error are present. One is the nonconstancy of the equilibrium constant in the range of measurement and the other is the possibility of overlapping of different reactions.

The first of these errors is due to the fact that the equilibrium constant used above is a concentration constant, and it changes whenever the activity coefficients of the compounds participating in the equilibrium are changed. This source of error may be neglected under the conditions in which reaction (2) was studied, as in this case the ionic strength of the medium had a high and constant value and the en concentration was only varied slightly in the range of measurement. In the case of reaction (1) conditions were less favourable since here the alkali concentration was varied and the ionic strength of the solutions could not be kept constant during the experiment. Therefore, the error may be appreciable in this case.

An attempt was made to reduce this effect by making measurements in concentrated salt media. However, the equilibrium constants in these solutions were too small to be accurately determined and this method was therefore of little value. Experiments were made both with NaNO_3 and NaClO_4 as the salt medium. The effect of the two salts was found to be exactly the same. In Figure 3 and in Table 5 results obtained with solutions of different NaNO_3 concentrations are shown. When salt (NaNO_3 or NaClO_4) was added to a neutral solution of Cu en_2^{2+} a small but definite change in the extinction of the solution was observed. The possibility of complex formation between Cu en_2^{2+} and the anion of the salt can therefore not be precluded. When such complex formation occurs, conditions are analogous to those that led to equations (12) and therefore at constant salt concentration (12) applies.

The error due to the overlapping of other reactions is also small in the case of reaction (2). In reaction (1) some overlapping by reaction (2) is obtained. However, making use of the determined values of the equilibrium constants k and k_1 , the extent of overlapping can be calculated and corrections can be applied to the experimentally determined δ -values. This can be effected quite generally using the equations

Table 6. Collection of values for the concentration complexity constants for copper complexes in alkaline ethylenediamine solutions.

No.	Complexity constant	k	$t^\circ\text{C}$	Medium	Method	References
1	$k = \frac{[\text{Cuen}^{2+}]}{[\text{Cu}^{2+}][\text{en}]}$	$10^{10.72}$	25°	1 M (KNO_3)	glass electr.	[3]
2	$k = \frac{[\text{Cuen}_2^{2+}]}{[\text{Cuen}^{2+}][\text{en}]}$	$10^{9.31}$	25°	1 M KNO_3	glass electr.	[3]
3	$k = \frac{[\text{Cuen}_3^{2+}]}{[\text{Cuen}_2^{2+}][\text{en}]}$	0.1	25°	1 M KNO_3	spectroph.	[3]
4	$k = \frac{[\text{Cuen}_2\text{OH}^+]}{[\text{Cuen}_2^{2+}][\text{OH}^-]}$	3	25°	0.08–0.2 M NaOH	spectroph.	This paper
5	$k = \frac{[\text{Cuen}(\text{OH})_2][\text{en}]}{[\text{Cuen}_2\text{OH}^+][\text{OH}^-]}$	$5 \cdot 10^{-2}$	25°	0.63 M NaOH	spectroph.	This paper
6	$k = \frac{[\text{Cu}(\text{OH})_4^{2-}][\text{en}]}{[\text{Cuen}(\text{OH})_2][\text{OH}^-]^2}$	$3 \cdot 10^{-4}$	25°	0.5 M NaOH	estimation	This paper

The last complexity constant in Table 6 is calculated by making use of the solubility data for cupric hydroxide in alkali as given in reference [6] ($[\text{Cu}(\text{OH})_4^{2-}]/[\text{OH}^-]^2 = 1.88 \cdot 10^{-3}$), the solubility data in Table 1 and the complexity constants 4 and 5 in Table 6.

$$\left. \begin{aligned} \varepsilon &= \varepsilon_1 \alpha_1 + \varepsilon_2 \alpha_2 + \sum_i \varepsilon_i \alpha_i, \\ 1 &= \alpha_1 + \alpha_2 + \sum \alpha_i. \end{aligned} \right\} \quad (21)$$

Here the index i refers to the interfering complexes. If the values of α_i and ε_i can be estimated the corrections in the δ - and δ' -values respectively can easily be calculated. In the case of reaction (1) corrections were made for the presence of $\text{Cuen}(\text{OH})_2$ and in the case of reaction (2) for the presence of $\text{Cu}(\text{OH})_4^{2-}$ and Cuen_3^{2+} . In Figures 3, 4 and 5 corrected δ - and δ' -values, respectively are used.

One further point is of interest in this connection. From the definition of δ and δ' it follows that their values at different wavelengths should be proportional. Considering the δ' -values calculated for the two different wavelength regions in Tables 3 and 4 some discrepancy in the proportionality is obtained. At low copper concentrations the ratios of the two δ' -values are fairly constant but at high copper concentrations this is no longer true, the deviations being significant in solutions with low en concentrations. A possible explanation is that in these solutions polynuclear complexes are formed. This is supported by the fact that the deviations appear in concentrated solutions and near the limit of precipitation of $\text{Cu}(\text{OH})_2$, both these conditions being favourable for the formation of polynuclear complexes. Also, the existence of polynuclear complexes of the form $\text{Cu}(\text{OH})_2\text{Cu}^{2+}$ in hydrolysed cupric solutions has recently been reported [9].

Considering the complex $\text{Cuen}(\text{OH})_3^-$, referred to earlier, it is obvious that the method used will not reveal its existence. In principle it could be detected by making

measurements at different OH^- concentrations and comparing the f -values obtained. However, as f will change rather strongly due to reaction (1) this approach would be difficult and was therefore not attempted.

Finally, in Table 6, a survey of the complexity constants for the different complexes existing in alkaline cupric-ethylenediamine solutions is given.

Summary

Complex formation in alkaline cupric-ethylenediamine solutions has been investigated by a spectrophotometric method. The existence of the complexes $\text{Cu en}_2\text{OH}^+$ and $\text{Cu en}(\text{OH})_2$ in these solutions has been established and their complexity constants determined. The reliability of the methods used is discussed.

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Studies on synthetic growth substances

IX. The configuration of N-arylamino-propionic acids with growth-regulating activity

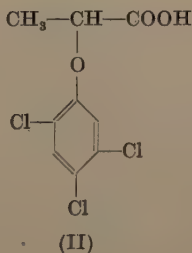
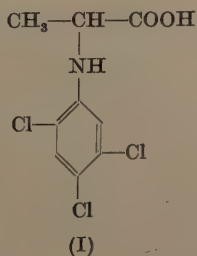
By ARNE FREDGA

With 2 figures in the text

It is known that N-arylamino-carboxylic acids may act as plant growth regulators; their biological activity is in general somewhat lower than that of the corresponding aryloxy-carboxylic acids. Some years ago, Matell and Åberg reported that the optically active forms of α -anilino-propionic acid have very different biological effect, the (+)-form being much more active than its antipode (1, 2). It was, however, not possible to determine the absolute configuration of the more active form (3). In the case of the aryloxy-carboxylic acids, the D-forms show the greater biological effect and Matell suggested that the same may hold good for the arylamino acids.

Attempts to resolve the α -[2-naphthylamino]- and the α -[2,4-dichloroanilino]-propionic acids into the optical antipodes met with no success (3). Owing to the amphoteric character of the arylamino acids, their tendency to give well-defined salts with optically active bases is rather low, and in most cases only oily products were obtained.

In search of a suitable arylamino-carboxylic acid, the present author has chosen the α -[2,4,5-trichloroanilino]-propionic acid (I). The derivatives of 2,4,5-trichloroaniline have in general high melting points and good crystallisation tendency. The three chlorine atoms could be expected to increase the acidity, thus favouring salt formation. The corresponding trichlorophenoxy-propionic acid (II) has strong biological effect, the D-(+)-form being by far more active. The racemic acid melts about 40° higher than the antipodes (4) and the melting-point diagram indicates the existence of a true racemic compound of great stability (5). The chances for obtaining a quasi-racemic compound with the acid I could thus be regarded as favourable.



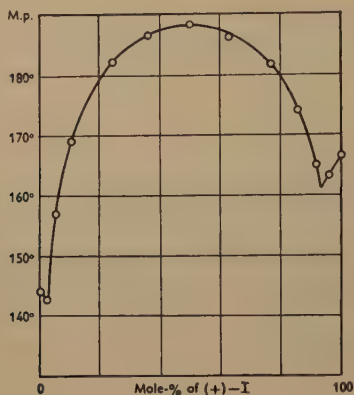


Fig. 1. (+)-I and (-)-II.
Quasi-racemic compound.

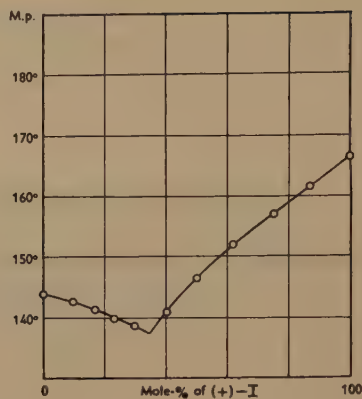
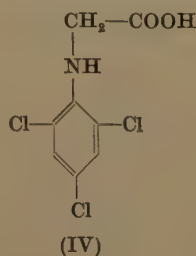
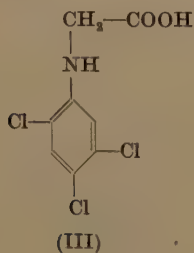


Fig. 2. (+)-I and (+)-II.
Eutecticum.

The 2.4.5-trichloroanilino-propionic acid (I), prepared from trichloroaniline and ethyl α -bromopropionate, fully came up to the anticipations. The resolution was easily performed with (+)- and (-)- α -phenyl-ethylamine, and the acid forms a very stable true racemic compound. The melting-point diagrams of the optically active forms of I and II give a very beautiful example of a quasi-racemic compound (Figs. 1 and 2), and it is obvious that the acids with opposite modes of rotation also have opposite configurations. The D-configuration of the (+)-2.4.5-trichloroanilino-propionic acid is thus established.

For comparison of the growth-regulating activity, the 2.4.5-trichloroanilino-acetic acid (III) and the 2.4.6-trichloroanilino-acetic acid (IV) have also been synthesised, using ethyl bromoacetate. In the latter case, the reaction proceeds rather slowly, obviously due to steric hindrance from the two Cl-atoms in ortho-position to the amino group. As could be expected, this steric effect is far more pronounced in the reaction between 2.4.6-trichloroaniline and ethyl α -bromopropionate, and a satisfactory procedure for preparing the 2.4.6-trichloroanilino-propionic acid has not been found as yet. Similar difficulties are not encountered in the preparation of 2.6-dichloro-substituted phenoxy acids.

The growth-regulating activity of the acids in question is being investigated by Professor B. Åberg (Royal Agricultural College, Uppsala). The results will be published elsewhere, but Professor Åberg has kindly given some preliminary data. The 2.4.5-trichloroanilino acids possess strong auxin activity, somewhat lower than the



activity of the corresponding trichlorophenoxy acids. There is a marked difference between the antipodes of the propionic acid derivative (I), the D-(+)-form being by far more active. The biological activity of the acetic acid derivative (III) is comparable to that of the dextrorotatory propionic acid.

On the other hand, the 2.4.6-trichloroanilino-acetic acid (IV) is a typical anti-auxin; it is comparable to 2.4.6-trichlorophenoxy-acetic acid which has been investigated by several authors (6, 7).

On the whole, there is a close resemblance in biological activity between the trichloroanilino acids and the corresponding trichlorophenoxy compounds. This indicates that the mechanism of the growth-regulating activity is the same or at least very similar. Considering the investigations of Matell on the aryloxy-carboxylic acids (3) and the results of the present investigation, it seems reasonable to assume that optically active α -N-arylamino-carboxylic acids having stronger auxin effect than their antipodes possess D-configuration. Perhaps it should be pointed out that these acids are derivatives of the "unnatural" D-amino acids.

Experimental

2.4.5-Trichloroanilino-acetic acid. 19.6 g (0.1 mole) 2.4.5-trichloroaniline and 8.4 g (0.05 mole) ethyl bromoacetate were heated to 160° for one hour. The product was dissolved in the necessary amount of alcohol and 7 g potassium hydroxide, dissolved in 100 ml water was added. The mixture was distilled with steam until the greater part of the unchanged trichloroaniline had passed over; the rest was extracted from the alkaline solution with ether. On acidification (to pH = 3), 8.8 g of crude acid were obtained (69 % of the theoretical). It was recrystallised once from ethanol and three times from ethyl acetate (once with charcoal). The yield of pure acid was only 1.7 g but further quantities could be regained from the mother liquors. The repeated recrystallisations from ethyl acetate were necessary to obtain a colourless product. The acid forms short-prismatic crystals with m.p. 179.5–181.5°.

37.60 mg substance: 8.90 ml 0.0495 N NaOH.

$C_8H_6O_2NCl_3$ (254.5) Cl calc. 41.79 found 41.55

2.4.6-Trichloroanilino-acetic acid was prepared from 2.4.6-trichloroaniline in the same way as the isomer. The reaction mixture was, however, kept at 160–170° for 5 hours; in spite of that, the yield of crude product was lower. It was recrystallised twice from benzene and once from dilute ethanol. The acid forms glistening needles with m.p. 184.5–185.5°.

39.54 mg substance: 9.32 ml 0.0498 N NaOH.

$C_8H_6O_2NCl_3$ (254.5) Cl calc. 41.79 found 41.62

racem-2.4.5-Trichloroanilino-propionic acid. 39.2 g (0.2 mole) 2.4.5-trichloroaniline were heated with 18.1 g (0.1 mole) ethyl α -bromopropionate to 160–170° for 6 hours. The product was isolated as described above for the acetic acid derivative. Yield of crude acid 15.0 g (55 % of the theoretical). After one recrystallisation from ethanol and two from ethyl acetate, 8.7 g pure acid was obtained as short-prismatic, colourless crystals with m.p. 202–203.5°.

31.52 mg substance: 7.05 ml 0.0498 N NaOH.

$C_9H_8O_2NCl_3$ (268.5) Cl calc. 39.61 found 39.50

The acid is not quite stable. After standing for a few months (even in the dark) it assumes a yellowish colour and the smell of 2,4,5-trichloroaniline is easily perceptible.

Preliminary experiments on resolution. Optically active α -phenyl-ethylamine gave readily crystallising salts and very good resolution; the mode of rotation of the acid is opposite to that of the amine used. Levorotatory acid can also be isolated through the cinchonine salt. Cinchonidine and brucine yielded salt containing levorotatory acid of moderate activity. The acid regenerated from the quinine salt was quite inactive and the strychnine salt could not be brought to crystallisation.

Resolution of the racemic acid. 12.1 g acid (0.045 mole) and 5.4 g (–)- α -phenyl-ethylamine (0.045 mole) were dissolved in 100 ml of acetone and 100 ml of hot water was added. After standing in a refrigerator over-night, the salt was filtered off and recrystallised from dilute acetone. After each crystallisation, a small sample of the salt was decomposed, the acid liberated and the rotatory power determined in acetone solution.

Crystallisation	1	2	3
ml water	100	25	20
ml acetone	100	25	20
g salt obtained	6.8	6.1	5.4
$[\alpha]_D^{25}$ of the acid . . .	+34°	+35°	+34.5°

The mother liquor from the first crystallisation was evaporated and the acid isolated; 8.2 g having $[\alpha]_D^{25} = -22.8^\circ$ (acetone solution) was obtained. This acid was dissolved with 3.7 g of (+)- α -phenyl-ethylamine in dilute acetone and the salt was recrystallised as described above.

Crystallisation	1	2	3
ml water	30	30	25
ml acetone	30	25	20
g salt obtained	7.3	6.3	5.7
$[\alpha]_D^{25}$ of the acid . . .	–33°	–34.5°	–34°

(+)-2,4,5-Trichloroanilino-propionic acid. The (–)-phenyl-ethylamine salt (5.4 g) was decomposed with sulphuric acid (acidification to pH = 3) and the organic acid was extracted with ether. It was recrystallised once from ethyl acetate + light petroleum and once from dilute (45%) ethanol. The yield of crude acid was 3.6 g; after the recrystallisations there remained 2.7 g. Small glistening needles with m.p. 166–167°.

29.79 mg substance: 6.66 ml 0.0942 N NaOH.

$C_9H_5O_2NCl_3$ (268.5) Cl calc. 39.61 found 39.48

0.2286 g substance dissolved in acetone to 10.00 ml: $2\alpha = +1.57^\circ$. $[\alpha]_D^{25} = +34.3^\circ$; $[M]_D^{25} = +92.1^\circ$.

(–)-2,4,5-Trichloroanilino-propionic acid. The (+)-phenyl-ethylamine salt (5.5 g) was decomposed as described above and the acid was recrystallised once from benzene and once from dilute ethanol. Yield of crude acid 3.8 g; after recrystallisation 3.4 g. M.p. 166–167°.

28.65 mg substance; 6.40 ml 0.0942 *N* NaOH.

$C_9H_8O_2NCl_3$ (268.5) Cl calc. 39.61 found 39.59

0.2384 g substance dissolved in *acetone* to 10.00 ml; $2\alpha = -1.63^\circ$. $[\alpha]_D^{25} = -34.2^\circ$; $[M]_D^{25} = -91.8^\circ$. — 0.2005 g substance dissolved in abs. *ethanol* to 10.00 ml; $2\alpha = -1.145^\circ$. $[\alpha]_D^{25} = -28.6^\circ$; $[M]_D^{25} = -76.7^\circ$. — 0.1816 g substance dissolved in *chloroform* to 10.00 ml; $2\alpha = -1.095^\circ$. $[\alpha]_D^{25} = -30.1^\circ$; $[M]_D^{25} = -81.0^\circ$.

The melting point diagrams were determined by Rheinboldt's method (8), using an apparatus described by Friedrichs (9). The samples were stirred in the capillary with a thin glass rod; in this way the melting points for pure compounds are generally found about 0.5° lower than by the ordinary method. The mixtures were prepared by dissolving weighed quantities of the components in 0.5–1 ml pure ether. After spontaneous evaporation of the solvent, the residue was powdered and dried over anhydrous calcium chloride. The data are given in Tables 1–2.

Table 1.

(+) -I and (–) -II

Mole-% (+) -I	M.p.
0.0	144.0°
2.2	142.7°
5.4	157.0°
10.3	169.0°
24.3	182.3°
35.8	186.5°
50.0	188.5°
62.7	186.0°
76.6	181.5°
85.5	174.0°
91.6	165.0°
95.9	163.2°
100.0	166.5°

Table 2.

(+) -I and (+) -II

Mole-% (+) -I	M.p.
0.0	144.0°
9.9	142.8°
16.9	141.5°
23.0	140.0°
29.9	138.8°
40.1	141.0°
50.2	146.5°
62.0	152.0°
75.3	157.0°
87.0	161.5°
100.0	166.5°

Summary

2.4.5-Trichloroanilino-propionic acid has been prepared and resolved into the optically active forms; the rotatory power found was $[\alpha]_D^{25} = +34.3^\circ$ and -34.2° respectively (acetone solution). The (+)-acid forms a quasi-racemic compound with L-(–)-2.4.5-trichlorophenoxy-propionic acid. The D-configuration of the (+)-trichloroanilino acid is thus established.

2.4.5- and 2.4.6-trichloroanilino-acetic acid have also been prepared and some preliminary experiments on the growth-regulating activity of the different acids are reported. The D-form of the propionic acid derivative is by far more active than the L-form.

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Laboratory of Organic Chemistry, Chemical Institute, University of Uppsala, November 1956.

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Studies on cupriethylenediamine solutions of cellulose

By HANS VINK

With 8 figures in the text

During the course of current investigations on the solution properties of cellulose in cupriethylenediamine (shortened CED) it was observed that these solutions could be extensively diluted with water without the cellulose being precipitated. Since the properties of these solutions do not seem to have been investigated previously, and since in many respects they offer some advantage over the more concentrated solutions, a systematic study of them was undertaken. In these diluted solutions the solvent no longer dissolves cellulose and they can be considered as thermodynamically unstable. However, the recombination of the dissolved cellulose proceeds so slowly that during the short period of time necessary for ordinary physico-chemical measurements, the solutions can be considered as fully stable.

When the dilution is continued, cellulose is ultimately precipitated. This occurs at lower degrees of dilution if the cellulose concentration in the original solution is high and if the cellulose has a high DP. As an example it can be stated that a rather concentrated (about 2 g/100 ml) solution of cellulose with $DP \sim 1000$ in 0.5 *M* CED could be diluted up to 15 times giving solutions that remained practically unchanged for several weeks. When dilutions are made with dilute alkali solutions instead of water the stability of the solutions is greatly increased.

In this paper mainly macromolecular properties of these solutions are considered. Measurements of viscosity, sedimentation and diffusion have been carried out. As CED solutions of cellulose are sensitive to oxygen, which causes oxidative degradation of the cellulose, this effect has been more closely studied and subsequent measurements were carried out in nitrogen atmosphere in order to minimize the degradation. The solutions in the dissolution vessels and the measuring apparatus were freed from oxygen by alternate evacuation and filling with nitrogen.

Materials

For this study two wood cellulose samples were used. One of them was a refined acetylation grade pulp having an α -cellulose content of 94.6 % (CCA-7) and a viscosity of 22 cp (Tappi 206 m). This was first extracted with organic solvents and then with water and was dried in air at 60°C. Here it is designated by B. The other was an unbleached sulphite pulp with a viscosity of 195 cp (Tappi 206 m). This was first bleached with sodium chlorite using mild conditions and then extracted with a 5 %

sodium hydroxide solution in nitrogen atmosphere at 100°C during 5 hours. After washing with water it was dried in air at 60°C. This cellulose is here designated by K. The cellulose samples were kept in airtight vessels and their water content was determined by drying in an oven at 105°C to constant weight.

The CED solution used was prepared according to Tappi [1]. The copper concentration of the stock solution was determined electrolytically and it was $c_{\text{Cu}} = 0.490 \text{ M}$. The alkalinity of the solution was determined by potentiometric titration and this gave for the ratio between the en- and copper concentrations $c_{\text{en}}/c_{\text{Cu}} = 1.93$. The stock solution was kept in an atmosphere of nitrogen.

The nitrogen gas used had been purified according to Wartenberg [2]. Its oxygen content was stated to be lower than $10^{-4} \%$.

Viscosity measurements

Viscosity measurements were carried out with solutions having the following copper concentrations: 0.49 , $\frac{0.49}{2}$, $\frac{0.49}{6}$ and $\frac{0.49}{12} \text{ M}$. The viscometer used was a reversal type Ubbelohde viscometer a photograph of which is shown in Fig. 1. During a set of measurements it formed a closed system and could easily be kept free from oxygen. The dimensions of the instrument are given in Table 1.

First, the oxidative degradation of cellulose in the CED solutions was investigated. The solutions were kept saturated with atmospheric oxygen by bubbling air through the solutions in the viscometer. The course of the degradation is illustrated in Fig. 2.

If a solution that was originally saturated with atmospheric oxygen was allowed to stand undisturbed in the viscometer for some time it was found that in solutions with high copper concentration the degradation rate diminished. This can be explained by the fact that CED itself is oxidized by oxygen and that the oxygen concentration in the solution is thereby diminished. When the solution was again saturated with oxygen by bubbling air through it the degradation assumed its original rate (curves 2 and 3 in Fig. 2). In Table 2 the degradation rate for the different CED solutions is given, expressed in per cent of the original value of η_{sp} per hour. This rate was almost independent of the cellulose concentration in the concentration interval investigated.

When the measurements were carried out in nitrogen atmosphere the degradation was very small. For the determination of the intrinsic viscosity of cellulose, measurements were made in nitrogen atmosphere and a correction was applied for the minute degradation that was still present. Alternatively, one could keep the solution saturated with atmospheric oxygen and make an extrapolation to zero time. The error was in this case somewhat greater but the results are in agreement with those obtained in nitrogen atmosphere. In Fig. 3 viscosity curves for the two cellulose samples and for solutions with different copper concentrations are shown.



Fig. 1. Photograph of the viscometer. The measuring bulb is filled by removing the instrument from the thermostat and inverting it.

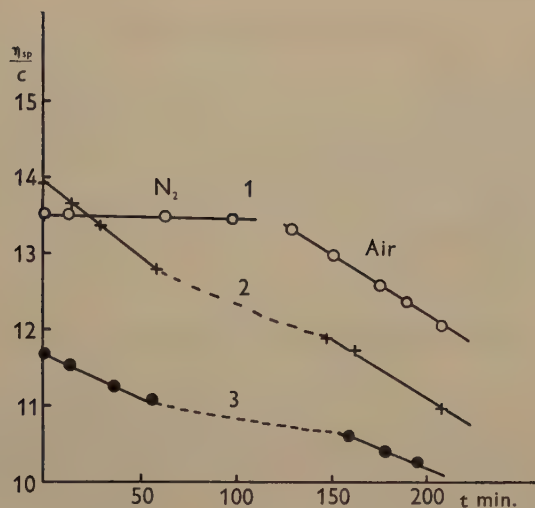


Fig. 2. Oxidative degradation of cellulose (sample K). Curves 1, 2 and 3 represent solutions that are resp. $\frac{0.49}{6}$, $\frac{0.49}{2}$ and $0.49 M$ with respect to CED. In 2 and 3 the solutions were kept unstirred in the dotted portions of the curves. The time of a measurement is taken as the time when the meniscus passed the upper mark of the viscometer.

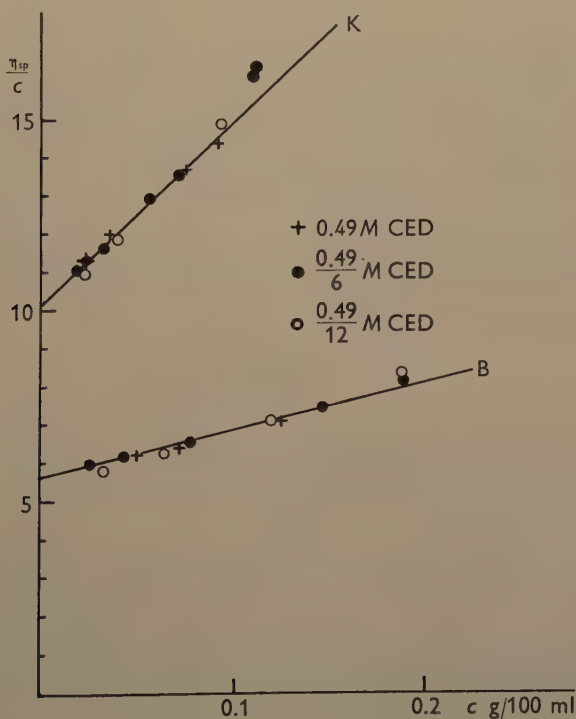


Fig. 3. Reduced viscosity curves.

Table 1. Viscometer data.

Capillary diameter	$d = 0.50$ mm
Capillary length	$l = 22$ cm
Efflux volume	$V = 4.47$ ml
Flow time for water at 25°C	226.9 sec.
Mean velocity gradient at 25°C: ¹	
for water	1070 sec ⁻¹
for $\frac{0.49}{6}$ M CED	1030 sec ⁻¹
for 0.49 M CED	840 sec ⁻¹

Table 2. Degradation of cellulose in CED.

$d\eta_{sp}/dt$ %/hour					
In solutions saturated with air					In N_2
C_{CED}	0.49 M	0.49/2 M	0.49/6 M	0.49/12 M	0.49/12-0.49 M
B	3.0	3.1	4.3	2.6	~ 0.1
K	6.8	8.2	8.5	6.6	~ 0.2

Molecular weight determination

It has been of great interest to carry out molecular weight determinations directly in these solutions. The method that seemed to be most suitable for this purpose was sedimentation together with diffusion. In earlier work this method had not been very successful. Gralén has carried out extensive investigations of the molecular weight of cellulose in Cuoxam and CED and found that the results obtained were 2-3 times higher than those determined by way of cellulose nitrate [3, 4]. Valtasaari and Hellman have later pointed out that at least part of the discrepancy is due to anomalous effects in the diffusion measurements [5].

Cellulose is present in CED solutions as a complex with copper. It is this complex that is the kinetic entity in the solution and that is accessible for observation. For the complex Svedberg's equation gives

$$M_x = \frac{RT s_x^0}{D_x^0 (1 - v_x \rho)^0} \quad (1)$$

The index x refers to the complex and indicates that it is unknown. s_x^0 and D_x^0 are the extrapolated values of the sedimentation and diffusion constants respectively and both are experimentally accessible. v_x is the partial specific volume for the complex. At present there are no methods for its experimental determination. Gralén circumvented this difficulty by using estimated values of v_x . First he determined the partial specific volume of cellulose. He then assumes that one copper atom is attached to each glucose unit in the cellulose molecule and that the partial specific volume of this copper is the same as in metallic copper. Combination of these two partial specific volumes gives the partial specific volume of the complex. In this way the molecular weight of the complex can be obtained and from that the molec-

¹ Calculated by Kroepelin's formula [6].

ular weight of the cellulose can be calculated. This approach is of course approximate.

In the present work it was found that it is possible to avoid this difficulty and by making certain assumptions the molecular weight of cellulose can be determined directly.

Let us assume quite generally that a substance with the molecular weight M in a certain solvent forms a complex with the molecular weight M_x . When the pycnometric method for the partial specific volume determination is used the following formula holds [7]:

$$1 - v_x \rho = \frac{1 - w_x}{A} \frac{dA}{dw_x} = \frac{1 - w_x}{A} \frac{dw}{dw_x} \frac{dA}{dw}, \quad (2)$$

where A = mass of solution in pycnometer

w = weight fraction of solute

w_x = weight fraction of complex

ρ = density of the solution.

For dilute solutions we have

$$\frac{w}{w_x} = \frac{M}{M_x} = \text{const.} \quad (3)$$

Hence
$$\frac{dw}{dw_x} = \frac{M}{M_x}. \quad (4)$$

Inserting Eq. (4) in Eq. (2) we get

$$\frac{M_x}{M} (1 - v_x \rho) = \frac{1 - w_x}{A} \frac{dA}{dw} \quad (5)$$

or at infinitesimal concentrations

$$\frac{M_x}{M} (1 - v_x \rho)^0 = \frac{1}{A} \left(\frac{dA}{dw} \right)^0. \quad (6)$$

On the other hand, for the molecular weight of the solute we have

$$M = \frac{M}{M_x} M_x = \frac{RT s_x^0}{D_x^0 \frac{M_x}{M} (1 - v_x \rho)^0}. \quad (7)$$

Thus, from Eq. (6) and Eq. (7) M can be obtained. In order to evaluate $(dA/dw)^0$ in Eq. (6), A is determined as a function of w and its derivative is determined at the point $w = 0$. This requires that the composition of the solvent for the complex is constant, independent of w . This can be accomplished if the solutions can be dialyzed and the assumption is made that the dialyzate represents the solvent for the complex.

This method is generally valid and can be used with the CED solutions of cellulose, provided that these solutions can be dialyzed.

It has been difficult to find suitable membranes for concentrated CED solutions. However, the diluted solutions can easily be dialyzed using ordinary cellulosic membranes and thus the above method could be applied for molecular weight determination in these solutions. These measurements were made in $\frac{0.49}{6} M$ CED solutions. The membranes were sufficiently resistant against these solutions and the electrolyte concentration was high enough to keep possible charge effects at a low value.

Dialysis experiments

Dialysis experiments, as well as sedimentation and diffusion experiments, were carried out in nitrogen atmosphere and at 25°C. As a cellulose membrane could absorb a considerable amount of copper it was equilibrated with the solvent before use. This was done by soaking it in the solvent for several hours. The duration of the dialysis experiments was about two days. This time was long enough for the equilibrium to be established, as was confirmed by extinction measurements. Thereby also information was obtained about the amount of copper bound by the cellulose.

In principle the best way of carrying out these experiments would be to dialyze several solutions of different cellulose concentration at the same time in the same solvent, as in this case the dialyzate would be identical for the different solutions. However, it was considered to be more practical to dialyze only one solution at a time and as in this case the dialyzates were not the same for solutions with different cellulose concentrations, the density measurements would give A and w values lying on curves displaced parallel to each other. Therefore, the A values were normalized by forming the difference

$$\Delta A = A - A_0 \quad (8)$$

where A_0 is the A -value of the dialyzate. Instead of A , ΔA was plotted as a function of w , and as $d\Delta A/dw = dA/dw$, $(dA/dw)^\circ$ could be determined. In Table 3 the primary values are given and in Fig. 4 a plot of ΔA against w is shown.

These measurements give $(M_x/M)(1 - v_x \rho)^\circ = 0.65$. When Gralén's method is used and when the number of copper atoms bound by a glucose unit is taken from Table 3, a value of 0.47 is obtained.

Table 3. Results of density measurements.

Cellulose	w	A_0 g	A g	ΔA g	No. of Cu atoms per glucose unit
K	0.00177	41.6165	41.6640	0.0475	0.547
B	0.00338	41.6033	41.6975	0.0942	0.541
B	0.00406	41.5920	41.6998	0.1078	0.535

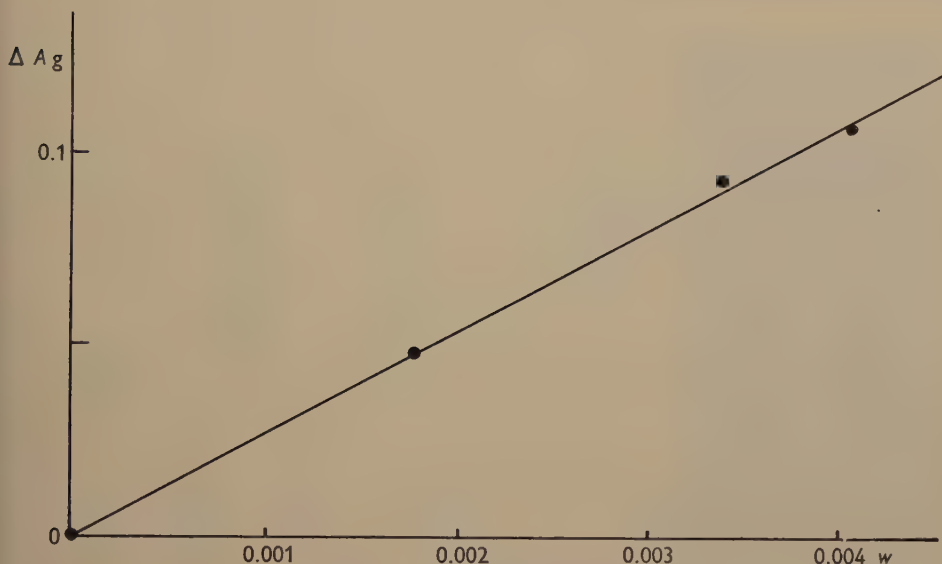


Fig. 4. Normalized density curve.

Diffusion measurements

In this case also the complex formation between cellulose and copper has to be taken into consideration. Some of the copper in the solution is bound by the cellulose and the solvent has to be diluted correspondingly. If this is not done the copper from the solvent will diffuse into the solution, the free diffusion of the cellulose complex is disturbed and too low diffusion constants are obtained. This has been treated by Valtasaari and Hellman [5].

In the present investigation the solvent has been compensated by diluting it with water to an extent corresponding to the amount of copper bound by the cellulose as given in Table 3. The measurements have been carried out in a glass cell of modified Svedberg type, a photograph of which is shown in Fig. 5. Primary values are given in Table 4 and in Fig. 6 the concentration dependence of the diffusion constants is shown.

Table 4. Diffusion constants for cellulose in $\frac{0.49}{6}$ M CED at 25°C.

B			K		
c g/100 ml	$D_A \cdot 10^7$	$D_m \cdot 10^7$	c g/100 ml	$D_A \cdot 10^7$	$D_m \cdot 10^7$
0.126	1.24	1.32	0.077	0.93	
0.170	1.42		0.179	1.10	1.23
0.290	1.52	1.63	0.235	1.28	1.45
0.409	1.66	1.89	0.245	1.21	1.48
			0.311	1.27	1.52

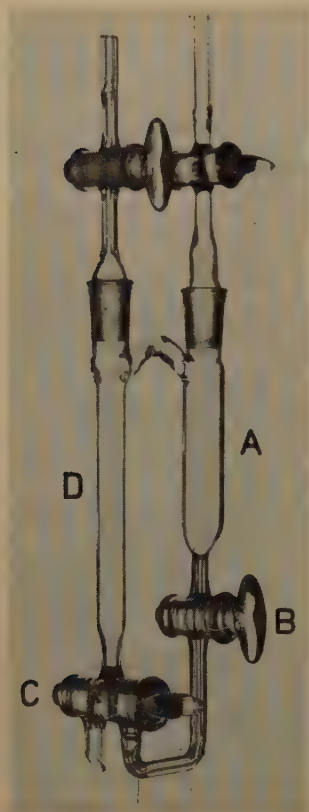


Fig. 5. Photograph of the diffusion cell. Solution is placed in column A and solvent in column D, the liquid level being higher in the former. The boundary is formed by means of stop-cock C, B being closed, and on opening B is moved to the center of D by the hydrostatic pressure.

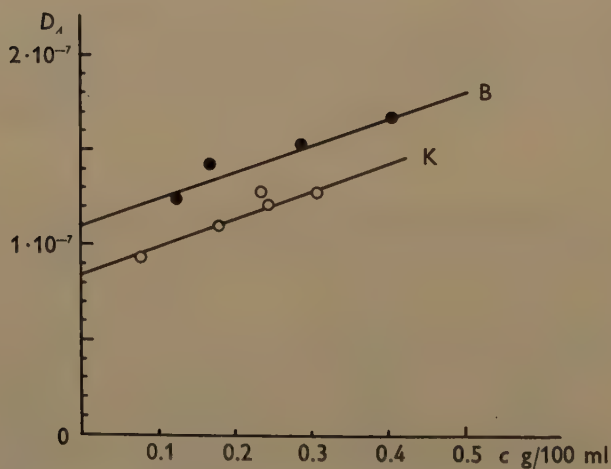


Fig. 6. Concentration dependence of diffusion constants.

Table 5. Sedimentation constants for cellulose in $\frac{0.49}{6} M$ CED at 25°C.

B		K	
c g/100 ml	$s \cdot 10^{13}$	c g/100 ml	$s \cdot 10^{13}$
0.076	4.25	0.0575	5.71
0.100	4.09	0.077	5.15
0.126	3.67	0.110	4.47
0.146	3.48	0.125	4.16
0.219	2.94	0.235	2.94
0.241	2.84		
0.409	2.16		



Fig. 7. Concentration dependence of sedimentation constants.

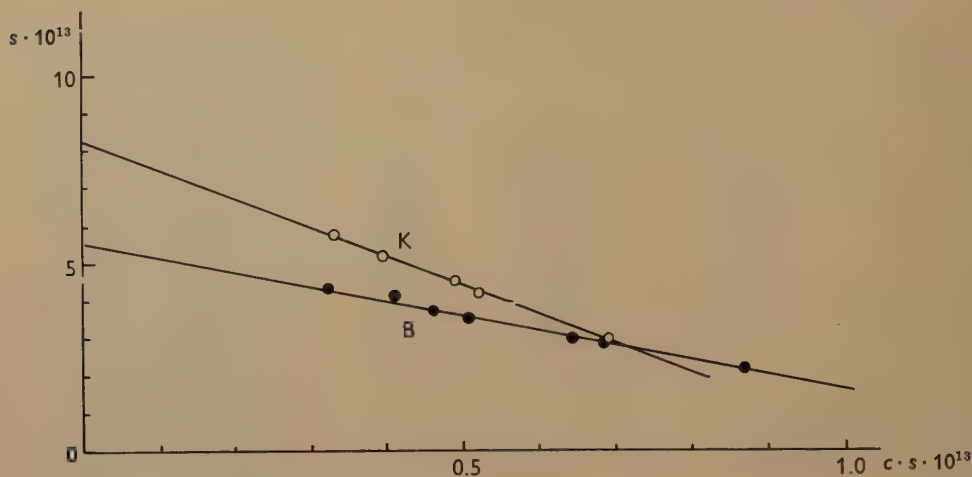


Fig. 8. Linear extrapolation of sedimentation constants to zero concentration.

Sedimentation measurements

The results of the sedimentation measurements are shown in Table 5 and Fig. 7. The concentration dependence of the sedimentation constant is often given by

$$s = \frac{s^0}{1 + kc} \quad (9)$$

A plot of s against cs will then give a linear extrapolation of the sedimentation constant. The result of this procedure is shown in Fig. 8.

Results

The results of the molecular weight determination are given in Table 6. For comparison molecular weights have also been calculated from the intrinsic viscosities using the viscosity equation

$$[\eta] = 0.0098 \times DP^{0.9}.$$

The constants in this equation are given by Marx [8]. They have been determined by way of cellulose nitrate by sedimentation and diffusion measurements.

Table 6. Results of molecular weight determination.

	s_{25}^0	D_{m25}^0	$\frac{M_x}{M} (1 - v_x \rho)^0$	M	DP	$[\eta]$	DP $_{[\eta]}$
B	$5.5 \cdot 10^{-13}$	$1.2 \cdot 10^{-7}$	0.65	175 000	1080	5.6	1150
K	$8.3 \cdot 10^{-13}$	$0.95 \cdot 10^{-7}$	0.65	333 000	2060	10.3	2270

Summary

CED solutions of cellulose can be diluted with water without cellulose being precipitated. These solutions are quite stable and can be used for physico-chemical measurements on cellulose. Viscosity, sedimentation, diffusion and dialysis experiments have been made. The molecular weight of cellulose has been determined and found to be in agreement with the value calculated from viscosity data using the modified Staudinger equation with constants given in the literature.

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Tryckt den 5 mars 1957

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Separation of folic acid and derivatives by electrophoresis and anion exchange chromatography

By E. USDIN¹ and J. PORATH

With 6 figures in the text

Methods for separation of folic acid from its derivatives which have previously been published (1, 2, 3, 4) have been applicable only for extremely small quantities—at most a few micrograms. Interest in separation of folic acid derivatives was aroused by the presence in human blood of a mixture of compounds having folic acid activity (5). One of us (J. P.) has developed methods of column electrophoresis (6) which can be used for very large quantities of material. Since folic acid derivatives in small quantities had been separated by paper sheet electrophoresis (7) attempts were made to separate these derivatives on large scale electrophoresis columns. Heinrich, Dewey and Kidder (8) have reported the use of a Dowex anion exchange column to separate pteroylglutamic acid from aminopterin. We therefore considered

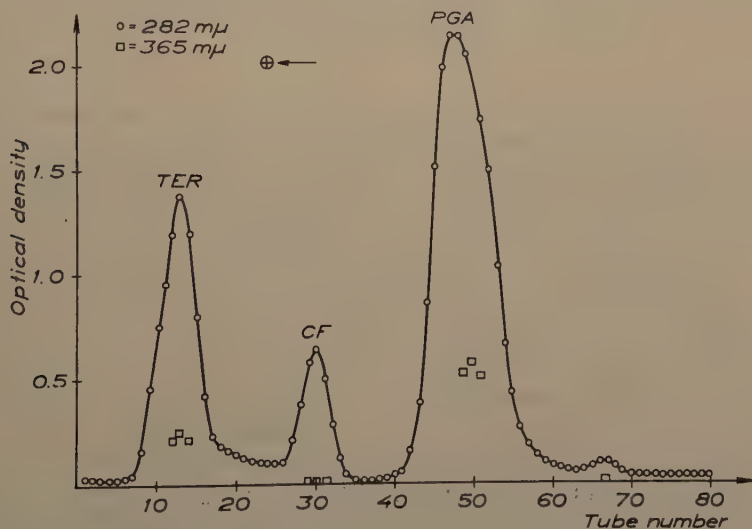


Fig. 1. Column electrophoresis separation of PGA (2.8 mg), TER (1.6 mg) and CF (0.3 mg). 5 ml/tube.

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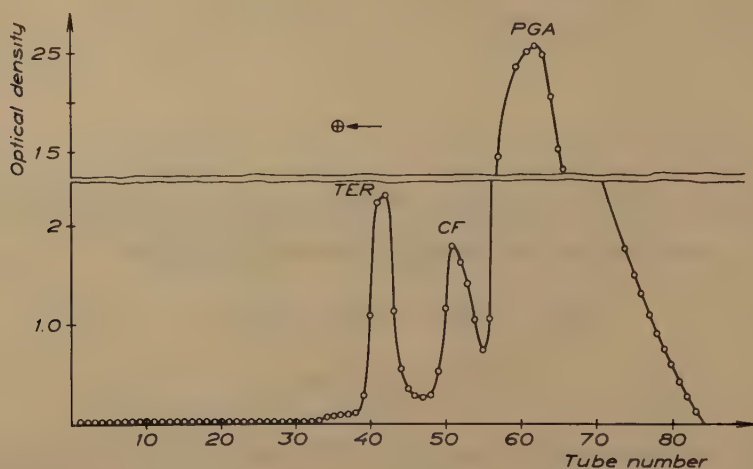


Fig. 2. Column electrophoresis separation of PGA (36.3 mg), TER (0.77 mg) and CF (0.60 mg). O.D. at 282 $m\mu$, 5.5 ml/tube.

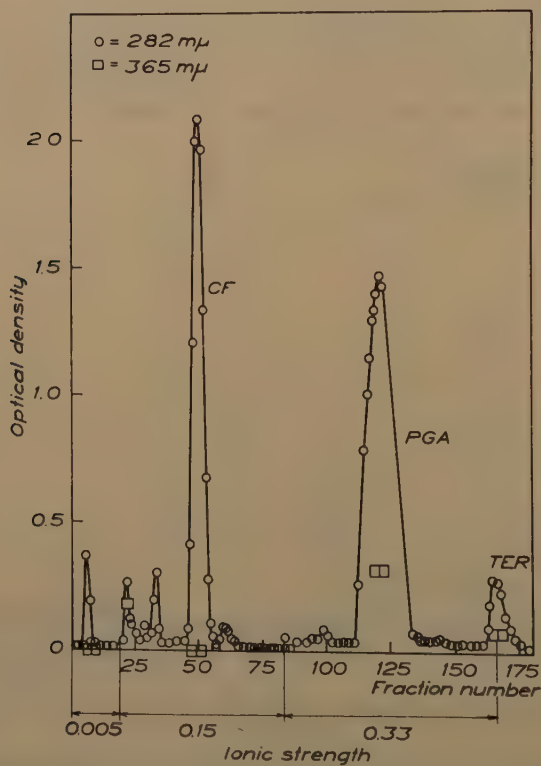


Fig. 3. TEAE-cell separation of PGA (1.12 mg), TER (0.77 mg) and CF (0.60 mg). 5.5 ml/tube.

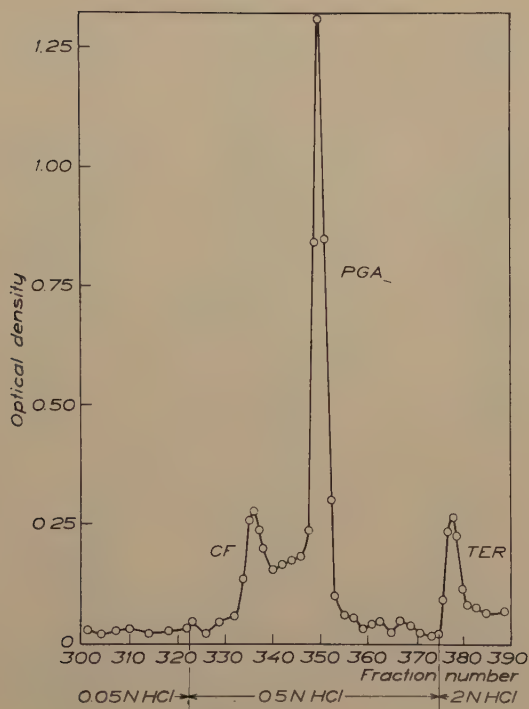


Fig. 4. Dowex-2 separation of PGA (1.12 mg), TER (0.77 mg) and CF (0.60 mg). Increasing concentrations of pH 6.1 phosphate buffer were used for the first 300 tubes but no 282 μ absorbing material was eluted. 5.5 ml/tube.

it to be of interest also to explore the possibility of using Dowex 2 and cellulose anion exchange columns for fractionation of similar mixtures.

Figure 1 shows the type of separation achieved with 2 ml of a mixture of 2.8 mg pteroylglutamic acid (PGA), 1.6 mg pteroyl triglutamic acid (teropterin, TER) and 0.3 mg N^{10} -formyl 5,6,7,8-tetrahydropteroylglutamic acid (citrovorum factor, CF) by column electrophoresis.

The column (2.0 $\times$ 150 cm) was prepared from Munktell cellulose powder in the manner previously described (9.5); sodium phosphate buffer of pH 6.1, ionic strength of 0.033 was used. The solution containing the mixture was displaced 25 cm from the top of column, a voltage of 2000 volts resulting in a current of 45 ma was applied for 12½ hours. All components moved towards the anode. Recovery was quantitative.

When disproportionate amounts of these same substances (26.3 mg PGA, 0.77 mg TER, and 0.60 mg CF) were applied in 2 ml, separation was still obtained, as illustrated in Figure 2. Microbiological assay with the three organisms, *Lactobacillus casei* (ATCC No. 7469) *Streptococcus faecalis* R (ATCC No. 8043) and *Leuconostoc citrovorum* (ATCC No. 8081) established incontrovertibly that the material contained in the first peak was pure TER, in the second CF and in the last PGA.

A triethylaminoethyl cellulose anion exchange powder (TEAE-cellulose) has been prepared (10), a sorbent similar to the DEAE-cellulose described by Peterson and Sober (11). Synthetic mixtures of folic acid and derivatives were placed on column

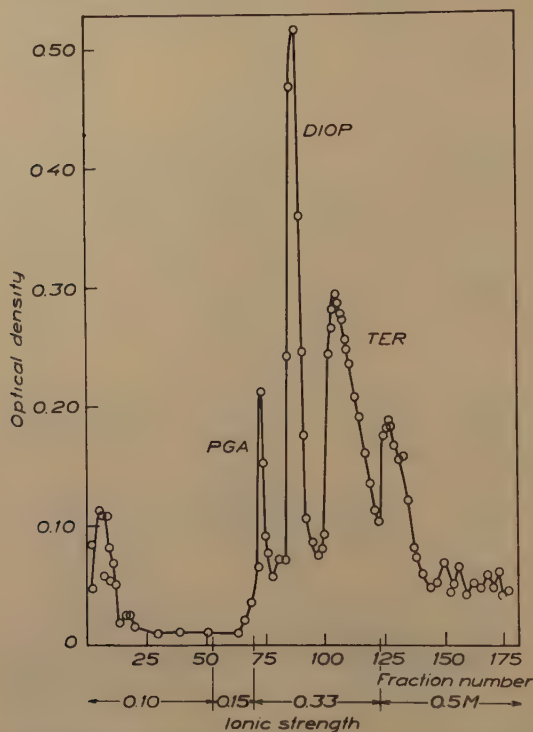


Fig. 5. TEAE-cell separation of PGA, DIOP and TER. The peak near the origin had no *S. faecalis* activity. 5.5 ml/tube.

(1.0 × 20 cm) of the free form of these exchangers and the columns were developed with increasing ionic strengths of phosphate buffer, pH = 6.1. Figure 3 illustrates a typical separation of a mixture of 1.12 mg PGA, 0.77 mg TER and 0.60 mg CF. In this and the succeeding experiments the material was applied in 20 ml and developed at a rate of 15 ml per hour. Since the material in the first (main) peak had no absorption at 365 m μ it was identified as CF. *Streptococcus faecalis* assay gave a negative result for the material in the last and was positive for the other two peaks, thus allowing complete identification of the materials.

When a column of Dowex 2 (1.0 × 20 cm) was used for a similar separation, no material was obtained with pH 6.1 phosphate developers even at 0.5 molar concentration. Elution was finally achieved with the use of 0.5 N and 2 N hydrochloric acid (Figure 4). At the pH required for desorption, many folic acid derivatives have been shown to be unstable (12).

Kazenko and Laskowsky (13) have shown that chicken pancreas enzyme removes only one of the glutamic acid residues from the triglutamate of pteric acid. A sample of 7 mg of TER was treated with 8 mg of Difco Bacto chicken pancreas in 1 ml of pH 6.1 phosphate buffer for four hours at 37°, then heated to boiling for one minute, cooled and filtered. Figure 5 illustrates the separation obtained on a TEAE-cellulose column. Microbiological assay confirmed the pteroyl diglutamic acid (DIOP) peak.

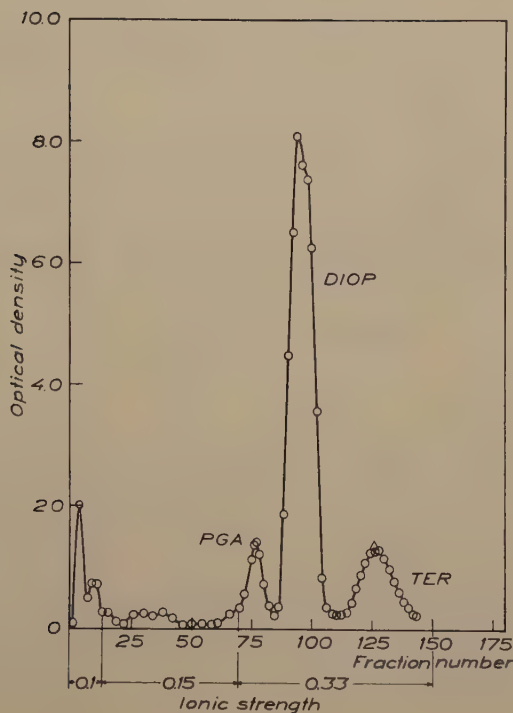


Fig. 6. Same conditions as Fig. 5, but larger sample, and longer time of incubation with enzyme.

A better preparation of DIOP was achieved when the enzyme was incubated for 20 hours with TER (Figure 6).

It is especially interesting that TER, CF and PGA are electrophoretically separated in a different order than by anion exchange chromatography. It can therefore be expected that complicated natural mixtures containing folic acid and its derivatives would be effectively fractionated by chromatography and subsequent electrophoresis (or *vice versa*).

The fractionation methods described in this paper are now being applied to purified material from human blood.

ACKNOWLEDGEMENTS

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Inhibition of yeast invertase (saccharase) by metal ions

III. Inhibition by Ag^+ and the complex formation between Ag^+ and imidazole derivatives

By KARL MYRBÄCK

With 4 figures in the text

Yeast invertase is very strongly inhibited by Ag^+ ions. The inhibition is completely reversible and non-competitive. It is dependent on the pH of the solution; the inhibition-pH-curves (obtained in experiments at a constant inhibitor concentration and at different pH values) are approximately parallel to the alkaline branch of the activity-pH-curve. This means that Ag^+ competes with H^+ for a group in the enzyme molecule. The alkaline branch of the activity-pH-curve has, as pointed out by Michaelis (1), the shape of a dissociation curve of a weak acid. This part of the activity-pH-curve is independent of the substrate concentration, i.e. the "acidic" group(s) that determine the alkaline branch of the curve are not involved in the combination enzyme substrate. Since the inhibition-pH-curves obtained with Ag^+ have the same form as the alkaline branch of the activity-pH-curve and are independent of substrate concentration it has been suggested (2) that Ag^+ (and certain other metal ions as Cu^{++} , Pb^{++} , Zn^{++} and Cd^{++}) are bound by the "acidic" group(s) postulated by Michaelis. This group is supposed to have a $\text{p}K_s$ value of about 6.7.

Yeast invertase is not known in the pure state. However, it seems safe to assume that the enzyme is a simple protein without any special prosthetic group. Consequently it will be assumed as working hypothesis that the Ag^+ -binding group which may be identical with the "acidic" group of Michaelis belongs to one of the common amino acids. This "acidic" group has $\text{p}K_s \sim 6.7$, and the simplest explanation therefore is that the group is the imidazole group of histidine; imidazole and imidazole derivatives generally have $\text{p}K_s$ -values between 6 and 7. It is also well known that imidazole, histidine etc. give complexes with heavy metals; many of these complexes have been isolated in pure form (3).

In our experiments we have tried to find out if the Ag-binding capacity of invertase can be ascribed to known groups in the amino acids as the imidazole group of histidine or free SH groups. If invertase has free SH groups, it must be expected that they bind Ag ions very strongly.

If it be supposed that the enzyme molecule has one group with $\text{p}K_s \sim 6.7$ and that this group binds one Ag ion then we have

$$\frac{[\text{Ag}^+][\text{E}^-]}{[\text{AgE}]} = K_{\text{Ag}}.$$

The dissociation constant of the silver complex, K_{Ag} , is supposed to have a value of about 10^{-8} (2, 4).

Experimental

Enzyme: Partly purified preparation from baker's yeast, If = 2 (5).

Assay mixture:

4.75 g saccharose

1 ml acetate buffer (*M* sodium acetate)

Silver nitrate solution and other additions

Water to 99 ml

1 ml enzyme solution.

All experiments were carried out at 30.0° C. Samples were removed at suitable times and pipetted into an equal volume of *N* sodium carbonate solution. Optical rotation was determined. Reaction times were selected to give 4–6 determinations between 0 and 30 % hydrolysis. In this range the conversion is practically proportional to reaction time; if the rotation is plotted vs. time a straight line is obtained the slope of which gives a true value of the initial reaction velocity. In the following the reaction rates (means of 4–5 determinations) are given as "relative activity", i.e. the activity referred to the activity of the non-inhibited enzyme at optimum pH (4.5). This maximum activity is put = 100.

All reagents were of analytical grade and redistilled water was used throughout.

The Ag-binding capacity of amino acids and certain other substances was determined; two different methods were used. In some cases Ag⁺ concentrations were measured electrometrically by determination of the potential between two pure silver electrodes, one in silver nitrate solution, the second in the same silver nitrate solution with addition of the substance to be tested (6). Most determinations of the Ag-binding capacity of different substances were, however, carried out in the following way: The relative activity of the enzyme at a certain Ag⁺ concentration and at a defined pH was measured. In parallel experiments the relative activity was determined at the same Ag⁺ concentration and pH in the presence of amino acids etc. If the added substance binds measurable amounts of Ag⁺ less enzyme is inactivated and a higher relative activity is found.

Results

Reactivation experiments were carried out with amino acids of different types (Table 1).

It is seen from Table 1 that of all amino acids investigated only the sulphur-containing acids and histidine have any detoxicating action, i.e. deionize silver in the concentrations applied. Glutathione (and other substances with free SH-groups) have an extremely strong action: in the mixture containing 5×10^{-6} *N* Ag⁺ and 5×10^{-6} *M* glutathione no inhibition of the enzyme occurred, i.e. there are practically no free Ag ions left in the solution. Methionine has an appreciable action, but only at considerably higher concentrations. It appears from the table that the complex formation between Ag⁺ and histidine or the sulphur-containing amino acids is instantaneous: incubation of the components does not increase their detoxicating action.

Neither orthophosphate nor glucose-6-phosphate in low concentrations have any appreciable capacity to deionize Ag (Table 2). As expected soluble bromides and (in higher concentration) chlorides reactivate the Ag-inhibited enzyme.

The detoxicating action of histidine was determined at various pH's (Table 3). Buffer 0.01 *M* sodium acetate + necessary amounts of acetic acid. pH was determined with the glass electrode in the reaction mixtures.

Table 1. $5 \times 10^{-6} N$ Ag⁺. pH = 5.3. Substances to be tested were added to mixture of water, buffer, AgNO₃ solution and substrate. Enzyme was added after a certain incubation time at 30°.

Addition		Incuba- tion (min.)	Relative activity	Addition		Incuba- tion (min.)	Relative activity
Substance	Molarity in mixture			Substance	Molarity in mixture		
—	—	—	22.9	Tryptophane	4×10^{-3}	30	24.1
α-Alanine	8×10^{-3}	1	21.7	Proline	5×10^{-3}	1	23.0
	"	30	24.0	Hydroxipro- line	5×10^{-3}	1	22.3
β-Alanine	2×10^{-4}	1	24.0	Histidine	2×10^{-3}	1	44.5
	"	10	22.9		"	30	45.4
	"	30	22.9		4×10^{-3}	1	67.8
	8×10^{-4}	1	23.8		"	30	67.8
	2×10^{-3}	1	23.8		8×10^{-3}	1	75.9
	8×10^{-3}	1	20.9		"	30	75.9
Serine	10^{-3}	1	23.0				
	9×10^{-3}	1	22.7				
Arginine	2×10^{-4}	30	20.9	Reduced Glu- tathione	5×10^{-8}	1	24.1
	10^{-3}	30	20.0		5×10^{-7}	1	29.5
	8×10^{-3}	1	25.0		10^{-6}	1	39.0
	"	10	22.9		2×10^{-6}	1	59.1
	1.6×10^{-2}	1	25.0		5×10^{-6}	1	100
	"	10	20.9				
Glut. acid	2.5×10^{-3}	1	22.0	Methionine	2×10^{-4}	1	33.0
Tyrosine	2×10^{-4}	1	22.9		"	30	33.3
	"	10	23.9		10^{-3}	1	58.3
	"	30	22.9		"	30	58.3
Tryptophane	10^{-3}	1	25.8		2×10^{-3}	30	75.8
	"	30	24.1		5×10^{-3}	30	90.8
	4×10^{-3}	1	25.8				

Table 2. $5 \times 10^{-6} N$ Ag⁺. Incubation time 1 min.

Addition		pH	Relative activity
Substance	Molarity in mixture		
—	—	4.5	58.9
Orthophosphate	5×10^{-3}	4.5	62.9
	2.5×10^{-2}	4.5	66.2
—	—	5.7	3
Orthophosphate	2.5×10^{-2}	5.7	3
—	—	5.4	7
Glucose-6-phosphate	10^{-3}	5.4	5
—	—	5.3	20.1
KBr	5×10^{-6}	5.3	95.5
NaCl	2×10^{-3}	5.3	95.3

It is seen from Table 3 and the corresponding Fig. 1, that the detoxicating action, i.e. the silver-binding capacity of histidine, increases very strongly with increasing

Table 3. 5×10^{-6} N Ag^+ . Incubation time 1 min.

Without histidine		Histidine added		
pH	Rel. activity	pH	[Histidine] $\times 10^3$	Rel. activity
4.43	83.3	4.82	2	68.1
4.82	61.9	4.82	4	77.2
4.84	58.4	4.82	8	89.6
5.04	45.8	4.82	8	89.6
5.28	28.2	5.48	1	23.7
5.30	23.7	5.31	2	41.7
5.34	27.1	5.31	2	41.7
5.68	8.3	5.57	2	45.4
5.70	6.0	5.30	2	44.5
6.02	2.5	5.28	4	70.5
6.70	0	5.26	8	90.9
		5.51	1	22.7
		5.69	2	45.4
		5.53	8	90.8
		5.92	2	27.3
		5.94	4	55.9
		5.97	8	81.8
		6.85	4	9
		6.82	8	32.7

pH: the action at pH 5.7 is very much greater than the action of the same total concentration of histidine at pH 4.8. This must mean that H^+ competes with Ag^+ for histidine. Experiments at $[\text{Ag}^+] = 10^{-5}$ support this conclusion (Table 4, Fig. 2).

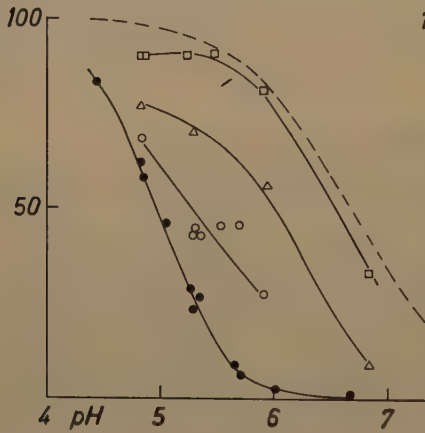


Fig. 1.

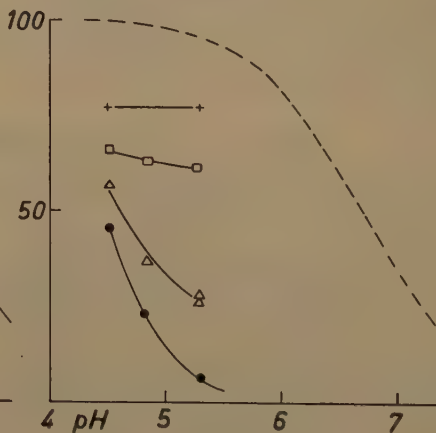


Fig. 2.

Fig. 1. Relative activity (ordinate) at 5×10^{-6} N Ag^+_{tot} and different histidine concentrations vs. pH., Activity-ph-curve; ●, no histidine; ○, 2×10^{-3} M histidine; △, 4×10^{-3} M histidine; □, 8×10^{-3} M histidine.

Fig. 2. Relative activity (ordinate) at 10^{-5} N Ag^+_{tot} and different histidine concentrations vs. pH., Activity-ph-curve; ●, no histidine; △, 4×10^{-3} M histidine; □, 8×10^{-3} M histidine; +, 16×10^{-3} M histidine.

Table 4. $10^{-5} N$ Ag^+ . Incubation time 1 min.

pH	[Histidine] $\times 10^3$	Rel. activity
4.52	—	45.5
4.52	4	56.4
4.52	8	66.0
4.52	16	77.3
4.84	—	23.2
4.84	4	37.7
4.84	8	63.2
5.30	—	5.9
5.30	2	11.4
5.30	4	28.6
5.30	4	27.3
5.30	8	61.3
5.30	16	77.3

In order to obtain a measure of the affinity of histidine for Ag^+ , e.g. at pH 5.3., we proceed in the following way. The dependence of the relative activity at pH 5.3 upon Ag^+ concentration is determined (Fig. 3). The shape of the curve indicates that a small amount of Ag^+ ($\sim 1.5 \times 10^{-6} N$) is bound strongly by impurities in the enzyme solution. From Fig. 1 we find the relative activities at $[Ag^+] = 5 \times 10^{-6}$ and different histidine concentrations (Table 5). The concentrations of Ag^+ necessary to produce these relative activities in the absence of histidine are found in Fig. 3 ($[Ag]$ in Table 5). If $1.5 \times 10^{-6} N$ Ag is supposed to be bound by impurities the concentration of Ag bound by histidine can be calculated ($[Ag \text{ bound}]$ in Table 5) and also the concentrations of free Ag^+ ($[Ag \text{ free}]$ in Table 5). The apparent dissociation constant K of the histidine- Ag compound is

$$K = \frac{[Ag \text{ free}] [Histidine]}{[Ag \text{ bound}]}$$

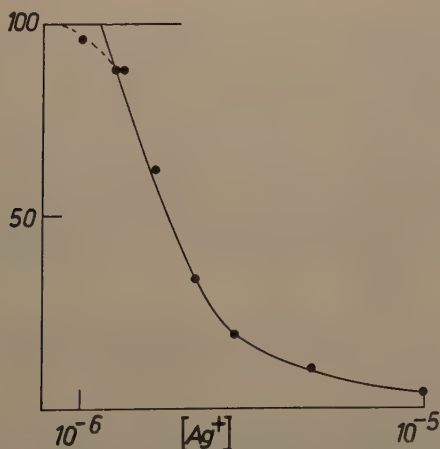


Fig. 3. Relative activity (ordinate) vs. Ag ion concentration. The same invertase preparation as in Figs. 1 and 2.

Table 5. Histidine at pH 5.3. $[\text{Ag}_{\text{tot}}^+] = 5 \times 10^{-6}$.

[Histidine]	Rel. act.	[Ag]	[Ag bound]	[Ag free]	<i>K</i>
2×10^{-3}	50	3.5×10^{-6}	1.5×10^{-6}	2.0×10^{-6}	2.7×10^{-3}
4×10^{-3}	75	2.5×10^{-6}	2.5×10^{-6}	1.0×10^{-6}	1.6×10^{-3}
8×10^{-3}	90	2.2×10^{-6}	2.8×10^{-6}	0.7×10^{-6}	2.0×10^{-3}

Table 6. Imidazole at pH 5.3. $[\text{Ag}_{\text{tot}}^+] = 5 \times 10^{-6}$.

[Imidazole]	Rel. act.	[Ag]	[Ag bound]	[Ag free]	<i>K</i>
—	25.0	—	—	—	
—	27.5	—	—	—	
8×10^{-3}	36.4	4.3×10^{-6}	0.7×10^{-6}	2.8×10^{-6}	32×10^{-3}
8×10^{-3}	35.5				
16×10^{-3}	44.5	3.8×10^{-6}	1.2×10^{-6}	2.3×10^{-6}	31×10^{-3}
16×10^{-3}					

and appears to have a value about 2×10^{-3} . However, since histidine has $pK = 6.3$ about 90 % must be present as the positive ion at pH 5.3 which means that the true dissociation constant of the silver compound is considerably lower than the apparent value, probably around 2×10^{-4} . It must be noted that these values are calculated under the assumption that 1 mole histidine binds 1 silver ion.

Since histidine had been found to complex Ag^+ , qualitatively in the same way as invertase, free imidazole was tested (Table 6).

The apparent dissociation constant of the imidazole-Ag compound is about 32×10^{-3} , i.e. about 15 times that of the histidine-Ag compound. This may mean that in the histidine-Ag compound not only the imidazole group is involved in the complex formation.

The histidine-Ag complex has also been studied electrometrically (4) with Ag electrodes in $5 \times 10^{-4} N \text{AgNO}_3$ and $0.05 M$ sodium acetate + acetic acid; one electrode vessel also contained histidine. pH was determined with the glass electrode. The concentration of free Ag^+ in the histidine-containing solutions was calculated in the conventional way. The values obviously include a small error depending on the Ag-complexing action of the acetate ion. This error does not vary with the pH of the solution. It was found that the complex formation histidine-Ag increases with increasing pH, showing that H^+ competes with Ag^+ for histidine. Further it was found that if a dissociation "constant" is calculated for a complex, supposed to contain histidine and Ag in the ratio 1:1,

$$K_1 = \frac{[\text{Ag free}] [\text{uncharged histidine}]}{[\text{Ag bound}]},$$

the values of K_1 have an order of magnitude of 2×10^{-4} , i.e. the same value as that found in the reactivation experiments. However, the K_1 values vary with pH, and for a certain pH they vary strongly with histidine concentration. But if it be assumed that in the complex the ratio of histidine to Ag is 2:1 the values of

$$K_2 = \frac{[\text{Ag free}] [\text{uncharged histidine}]^2}{[\text{Ag bound}]}$$

show a satisfactory constancy. The conclusion that one Ag ion binds two histidine molecules is in agreement with earlier investigations (3).

The capacity of certain *purine derivatives* to protect invertase against silver inhibition, i.e. their capacity to form Ag complexes, has been investigated. Many derivatives are too slightly soluble in water to allow determination by the enzymatic method.

Caffeine did not reactivate the Ag-inhibited enzyme (Table 7), nor had it any action on the non-inhibited enzyme. Thus, caffeine does not bind measurable amounts of Ag^+ at the concentrations used.

Table 7. Action of caffeine. pH = 5.4.

[Caffeine]	[Ag_{tot}^+]	Rel. activity
—	—	96.9
5×10^{-3}	—	96.5
—	5×10^{-6}	6.3
5×10^{-3}	5×10^{-6}	7.0

Table 8 shows corresponding experiments with adenine.

Table 8. $5 \times 10^{-6} N$ Ag^+ . Incubation at 30° .

pH	[Adenine]	Incubation (min.)	Rel. activity
4.5	—	—	65.5
	2.2×10^{-4}	5	80.8
	3.7×10^{-4}	1	80.8
	"	30	88.3
	7.4×10^{-4}	1	89.0
4.80	—	—	35.0
5.30	—	—	8.1
5.30	2.2×10^{-4}	1	20.6
5.35	"	30	24.6
5.38	3.7×10^{-4}	1	29.4
5.35	"	30	32.0
5.30	7.4×10^{-4}	1	57.0
	"	30	66.2
	3.7×10^{-3}	1	90.0
5.70	—	—	2.5
5.60	2.2×10^{-4}	1	14.0
	3.7×10^{-4}	1	26.5
	7.4×10^{-4}	1	41.9
	"	40	44.5
6.68	2.2×10^{-4}	1	11.0
6.50	3.7×10^{-4}	1	17.6
6.40	7.4×10^{-4}	1	26.8

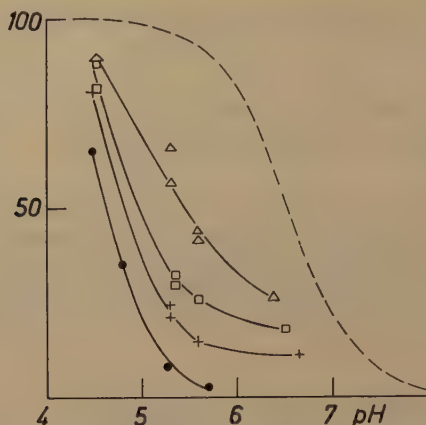


Fig. 4. Relative activity (ordinate) at $5 \times 10^{-6} N Ag_{tot}^{+}$ and different adenine concentrations vs. pH
 -----, Activity-pH-curve; +, $2.2 \times 10^{-4} M$ adenine; $\square$, $3.7 \times 10^{-4} M$ adenine; Δ , $7.4 \times 10^{-4} M$ adenine.

Table 8 shows that adenine has a very marked reactivating action of the Ag-inhibited invertase, and from Fig. 4 it appears that the Ag-complexing action of adenine increases with increasing pH in the range 4.5–6.5. Qualitatively the action of adenine is similar to that of histidine. Caffeine with its substituted imidazole ring has no action.

It seems that the action of adenine is not quite independent of the incubation time. The complex formation between Ag^{+} and adenine is not absolutely instantaneous which prevents calculation of a dissociation constant of the complex.

Discussion

Qualitatively the results on the complex formation between histidine and Ag^{+} are consistent with the assumption that the inhibition of invertase by Ag ions is caused by complex formation between the metal ion and imidazol groups of histidine in the enzyme protein. They are also consistent with the idea that the Ag-binding group of the enzyme is identical with the "acidic" group, assumed by Michaelis to be responsible for the alkaline branch of the activity-pH-curve of the enzyme. However, it is also clear that free histidine, not to speak of free imidazole, has a much lower affinity for the Ag ion than has the enzyme. Therefore, it seems that if the inhibition of invertase by Ag^{+} is caused by a reaction between Ag^{+} and imidazole groups, we have to conclude that more than one imidazole group, or one imidazole group together with adjacent groups of some other kind, are involved in the complex formation. It is conceivable that in the enzyme protein two imidazole groups have a position relative to each other that greatly facilitates complex formation.

SUMMARY

The "acidic" group of invertase which, according to Michaelis, determines the alkaline branch of the activity-pH-curve, is tentatively assumed to be the imidazole group of histidine. Earlier investigation of the enzyme inhibition by Ag^{+} had allowed the conclusion that Ag^{+} reacts with

the "acidic" group of Michaelis. It is shown by an enzymatic method and by direct electrometric determination of free Ag^+ in mixtures of AgNO_3 and histidine at various pH's that the formation of a histidine-Ag complex may explain the action of Ag^+ on invertase. Quantitative differences exist, however; the affinity of the enzyme to Ag^+ is much higher than that of histidine which, in turn, is much higher than that of imidazole.

The activity determinations have been carried out by Mrs. Ebba Willstaedt whose assistance is gratefully acknowledged.

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Some experiments concerning the effect of temperature on the crystallinity (orientation) in elongated neoprene specimens

By P.-H. LINDGREN

With 10 figures in the text

In connection with the previously reported X-ray investigation on the orientation in neoprene at different elongations (1), some experiments were also performed on the influence of an increase in the temperature upon the crystallinity in an elongated specimen.

Experimental

Fig. 1 gives a sketch of the apparatus and a photograph of some of its parts. An aluminium block, insulated with cork on the outside, was provided with channels through which liquid from a thermostat could be circulated. The specimen was clamped in a special holder which fitted into a groove in the aluminium block in such a way that the specimen was enclosed in a chamber, the bottom and side walls of which were formed by the aluminium block while the top and end walls were formed by the specimen holder. The side walls of the chamber had holes for the primary beam and for the scattered radiation. The diaphragm for the primary beam had a circular hole with a diameter of 0.8 mm and the direction of the primary beam was normal to the specimen.

The temperature in the thermostat bath was measured with a mercury thermometer and the difference in temperature between the chamber in the block and the bath was measured with a thermocouple (copper-constantan). One of the junctions was inserted into the groove through a narrow hole in the bottom to a point about 2 mm under the primary beam, the other junction was kept in the bath. There were also two other holes, usually stoppered, in the bottom of the groove which permitted temperature measurements nearer the ends of the chamber. The thermostat block is in its main features the same as that shown in a paper by Field (2). The temperature in the thermostat bath was controlled by a contact thermometer made in such a way that the adjustment of the temperature was accomplished by means of a screw which could be rotated by a magnet. This was driven by a synchronous motor and in this way the temperature of the bath could be slowly raised (or lowered) during the exposure.

The specimens were made in form of small ribbons, $20 \times 2 \times 0.5$ mm, as in the previously reported experiments (1). Two types of holders for the specimens were used (Fig. 1b). In one type the specimen was elongated with the aid of a screw with the thread in opposite directions on the two halves. In the other, the specimen was stretched by a weight. Due to the design of the other parts of the apparatus and also because of lack of space at the X-ray tube this holder was made for horizontal specimens. The load was transferred to the specimen by a cotton thread which was

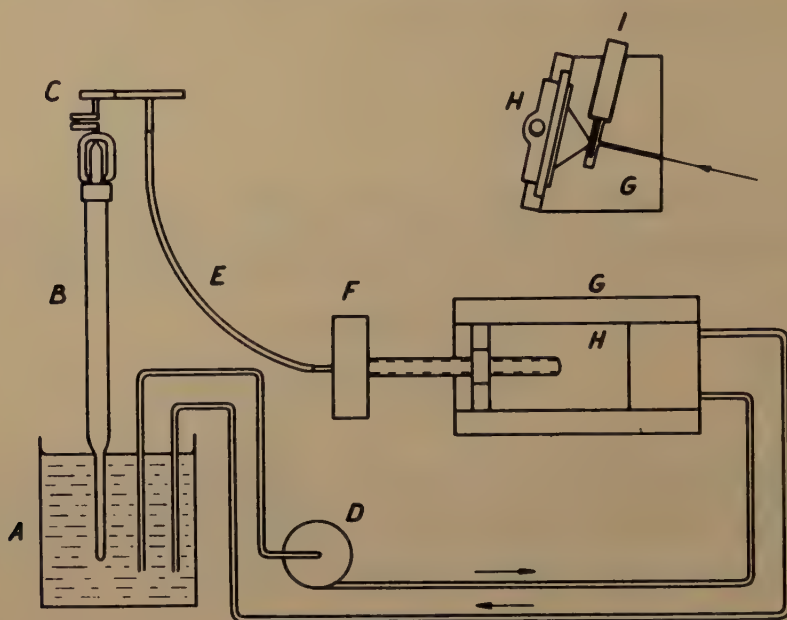


Fig. 1 (a). Sketch of the experimental arrangement. *A*, heating bath; *B*, contact thermometer; *C*, gear for rotation of the magnet; *D*, pump for driving the liquid (ethylene glycol) in the bath *A* through channels in the camera block *G*; *F*, synchronous motor moving the cassette *H* and turning the magnet at *C* by the flexible shaft *E*; *I*, specimen holder. (Fig. 1a from (4).)

passed over a small wheel, and fixed to a light metal bar to which the specimen was clamped. The bar was easily movable in a system of small wheels (Fig. 1b). This arrangement is not ideal because of the friction. In this case the force necessary to overcome it amounted to about 2 % of the smallest load applied and probably did not appreciably influence the results. Obviously the specimens in the first type of holder were heated under constant deformation and in the second type under constant load.

The scattered radiation was registered on a photographic film in a cassette which could be moved by the same motor which changed the temperature. The movement of the film was parallel to the direction of the fibre axis. Most of the scattered radiation was screened off from the film by a diaphragm (Fig. 1b). The equatorial maxima on the strongest interference circle, two small areas outside the maxima and two on the interference circle at a distance of about 45° from the equator were recorded on the film through some holes in the diaphragm. To simplify adjustment the holes corresponding to the interference circle were made elliptic, 1×1.5 mm, with the length normal to the fibre axis. The other holes which were used to give a background were circular and about 1 mm in diameter. In the middle of the diaphragm an arrangement with a slit for the primary beam and a covering slide was made to permit marking of the film. Apparatus with movable films have often been used and a camera designed for continuous recording of changes in the X-ray diagram with the temperature has been described by Stenhagen (3).

In the experiments an X-ray tube with copper anode and provided with nickel filter was used. The tension and current were respectively 40 kV and 20 mA. The temperature in the bath was raised 12°C per hour, the translation of the film was 12 mm per hour and the distance between specimen and film about 18 mm. At suitable time intervals (10 to 15 minutes) the film was

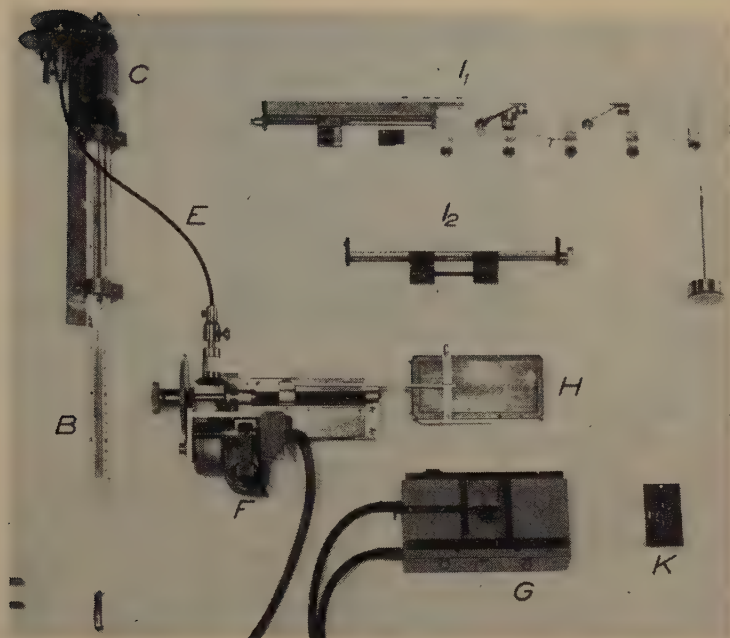


Fig. 1 (b). Photograph of some of the parts. K , diaphragm for the scattered radiation; I_1 , specimen holder for constant load; I_2 , specimen holder (with a specimen) for constant deformation. The other notations are the same as in (a).

exposed for a moment to the primary beam through the slit in the diaphragm and at the same time the temperature was measured. After exposure (usually 2 to 3 hours) and processing the film showed two groups of three traces corresponding to the holes in the diaphragm and in the middle a trace with marks corresponding to the primary beam (Figs. 2 and 3). The marks on the trace of the primary beam were only used to check that the displacement of the film had been continuous. From the readings of time and temperature a time-temperature curve for the specimen could be plotted (Fig. 3b), which gave the temperature in each point of the traces. From measurements on microphotometer recordings of the traces (Figs. 2b and 3c) curves could be obtained giving a relative value of orientation or crystallinity in the specimen (Figs. 2b and 3d).

It is seen in Fig. 3c that the recordings of the traces show many irregularities. Those having short periods are probably due to small irregularities in the film and to vibrations of the galvanometer in the microphotometer, the others depend mostly upon variations of the intensity in the primary beam but they may also be due to the specimen. However, the larger irregularities ought to change the intensities in both traces corresponding to the interference circle in the same way and should not appreciably influence the result.

The photometer deflections of the traces in each group of traces were denoted in the following way (Figs. 2 and 3): the background deflection S_1 , the deflection corresponding to the maximum S_2 and the deflection corresponding to the amorphous circle S_3 . The differences $(S_2 - S_3)$ and $(S_2 - S_1)$ are called a and b . From the microphotometer recordings in which the galvanometer vibrations were smoothed out, the value $100a/b$, which was used as a measure of the crystallinity, was plotted against temperature for a number of points for each of the groups of traces. The



Fig. 2 (a). An X-ray diagram of an elongated neoprene specimen; fibre axis horizontal.

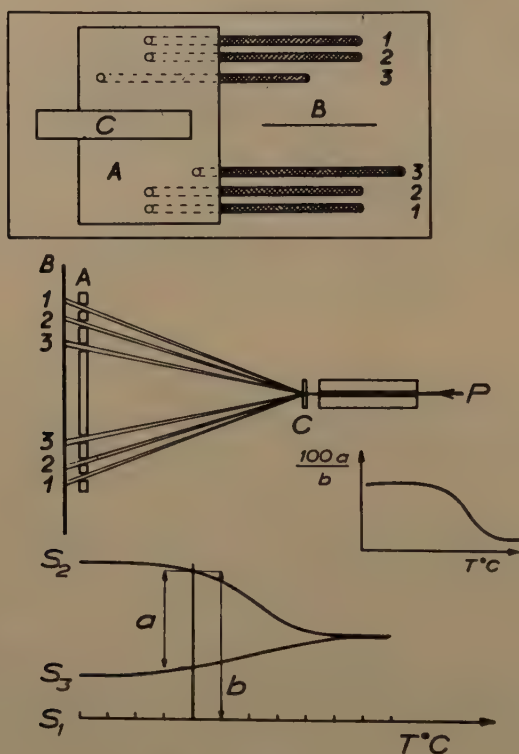


Fig. 2 (b). Sketches showing the relative positions of the specimen, the diaphragm for the scattered radiation and the film and schematic drawings of the microphotometer curves and of the calculated curve for the amount of crystalline material. *A*, diaphragm; *B*, film; *C*, specimen; *P*, primary beam. The numbers 1, 2 and 3 denote the scattered radiation and traces on the film corresponding respectively to the background, the maxima on the interference circle and to the recorded area on the interference circle. S_1 , S_2 and S_3 denote the corresponding microphotometer deflections, a is equal to $(S_2 - S_3)$, b equal to $(S_2 - S_1)$ and T is the temperature. $100a/b$ is the relative value for the amount of crystalline material used in this paper (Fig. 2b from (4)).

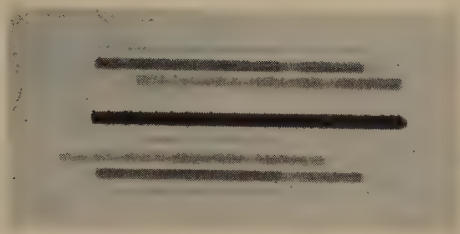


Fig. 3 (a). Print of a film with the traces of the continuously recorded scattered radiation; fibre axis horizontal and low temperature to the left. The specimen was neoprene B6 7, elongated 1000 % at 60°, cooled in ice-water and stored 48 hours at -4° . The thin traces parallel to the others and nearest the trace from the primary beam are due to small slits at the edges of the diaphragm. It is seen that the traces of the scattered radiation are shorter than the trace from the primary beam which shows that the specimen broke before the exposure was ended. At the right end of the traces from the scattered radiation weak shadows may be seen (at least on the film) which are due to scattering from the air.

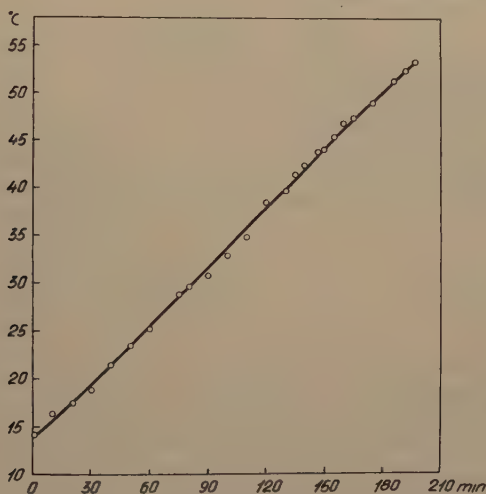


Fig. 3 (b). The time-temperature diagram for the exposure in (a).

readings corresponding to a group were connected by straight lines and from the broken lines an average of the value $100a/b$ for the two groups was determined at suitable temperature intervals. These averages were then plotted against the temperature (Figs. 2b and 3d).

It might be expected that there would be an increased tendency of the specimen to slip in the clamps at higher temperatures. Probably this effect did not cause considerable errors at constant deformations, at least it was not observed after the experiments that the specimen had changed its position noticeably or that its measured length had decreased more than a few per cent. The slip at the endpoints was larger at constant load than at constant deformation but could not be estimated in the same way as in the latter case.

In the experiments made at constant load the length of the specimen mostly increased during the exposure but the difficulty of measuring the slip also made measurements of the elongation during the heating correspondingly difficult. In these experiments one end of the specimen was

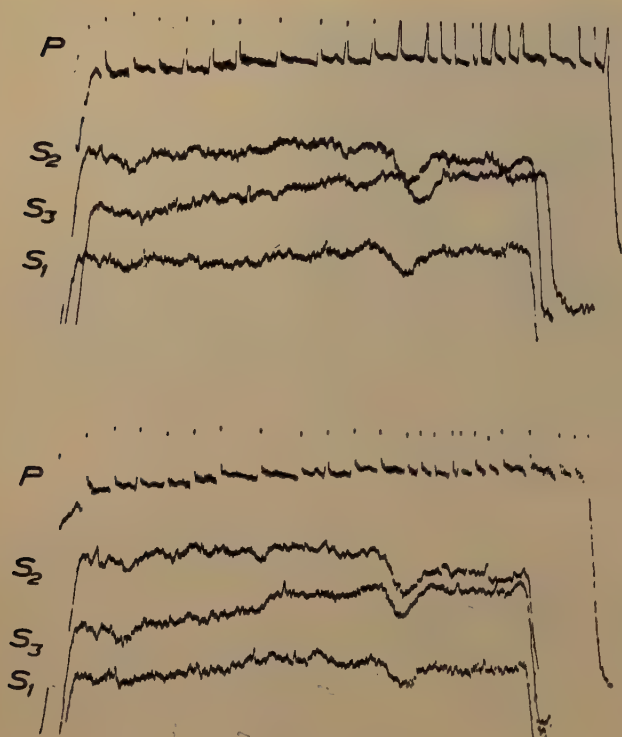


Fig. 3 (c). The microphotometer recordings of the film in (a) with low temperatures to the left. P , the recording of the trace from the primary beam and the notations S_1 , S_2 and S_3 the same as in Fig. 2b.

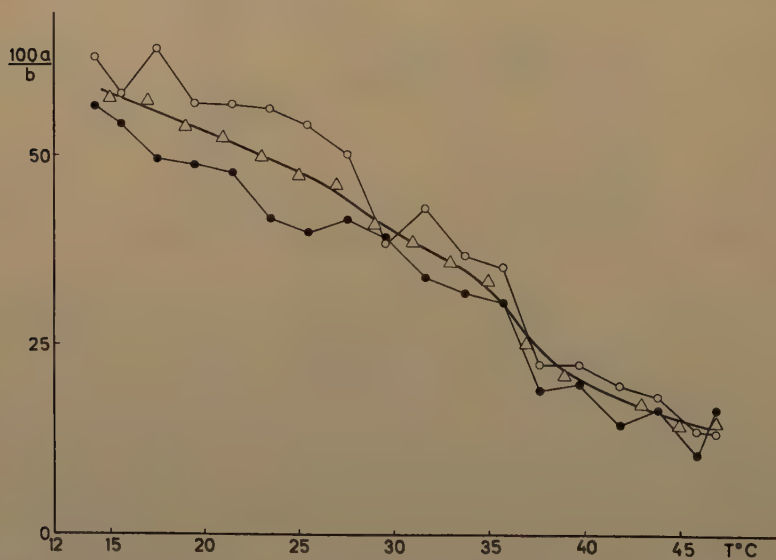


Fig. 3 (d). The final curve calculated from the microphotograms in (c). The circular dots are the values measured directly from the curves in (c) and the triangles give the linearly interpolated average values.

Table 1. The specimens investigated at constant deformation.

Unless otherwise stated the specimens were stretched at room temperature to an elongation of 500 %; *n* is the number of experiments.

Material	<i>n</i>	Temperature intervals	Remarks
(a) Unvulcanized materials			
ANE	2	24–51°; 30–35°	
ANG	1	22–62°	
E 15	1	26–62°	
B 23 _a	1	24–63°	
B 24 _a	1	24–63°	
Bö 7	1	22–54°	
Bö 17	1	24–55°	
Bö 22	1	24–54°	
Bö 7	1	24–47°	Elongated 1000 % at +60°, cooled in ice water and stored 48 hours at –4°.
Bö 17	2	16–39°; 14–45°	Elongation and cooling as above. Stored at –4° for 48 hours and 24 hours respectively.
(b) Vulcanized materials			
ANE	2	24–70°; 33–65–39°	Vulcanized normally. No recovery of the crystallinity was observed during the decrease of the temperature in the second case. Elongated 400 %.
ANE	1	29–71°	Curing time, min. 10
LB	3	18–51°; 23–48°; 20–50°	" " " 15
"	3	26–56°; 25–57°; 26–58°	" " " 20
"	2	25–63°; 30–63°	" " " 30
"	1	26–64°	" " " 40
"	2	28–60°; 30–61°	" " " 50
"	2	27–59°; 22–57°	" " " 60
"	3	32–57°; 25–57°; 25–55°	" " " "

Table 2. The specimens investigated at constant load.

In all these experiments the specimens were stretched at room temperature; *n* is the number of exposures.

Material	<i>n</i>	Elongation at the beginning of the experiment	Temperature intervals
(a) Unvulcanized materials			
ANE	3	10 %; 100 %; 250 %	20–56°; 21–50°; 24–57°
B 23 _a	1	100 %	24–45°
Bö 17	1	100 %	25–45°
Bö 22	1	100 %	23–44°
(b) Vulcanized materials			
LB 10*	1	350 %	25–59°
" 15	1	300 %	25–63°
" 20	4	100 %; 250 %; 500 % (2 exp.)	26–58°; 23–69°
" 30	2	250 %; 400 %	26–71°; 25–73°
" 40	1	350 %	24–53°; 24–61°
" 50	1	350 %	26–63°
" 60	1	350 %	25–61°
"			25–60°

* The figures give the curing time in minutes in the LB series.

at a fixed position, the other end moved and consequently different parts along the specimen scattered the radiation at different times. From the increase of the length of the specimen it follows that the volume of scattering material decreases and also that the load per unit cross-sectional area increases. Thus the expression "constant load" refers to the total load. No effects were observed which could with certainty be explained as due to the decrease of the amount of scattering material. In fact, with the specimens used, the absorption works against the influence of differences in thickness of the specimen.

In all experiments where the temperature was raised during the exposure the dependence of the scattering upon the temperature must also be involved but it has not been found possible to take this effect into account.

The materials investigated and the experimental conditions are given in Tables 1 and 2. The materials marked ANE and ANG were of American manufacture, all the others were made in Sweden. Of these E 15 is an emulsion polymer and the others are different bulk polymers.

The vulcanization was made according to the following formula:

100	parts of polymer
10	" " MgO
10	" " ZnO
2	" " phenyl- β -naphthylamine
5	" " kolophonium
1	part " sulfur

During the vulcanization the temperature was kept at 145° and the normal vulcanization time was 45 minutes. All temperatures in this paper are given in °C.

Results

(a) *The experiments at constant deformation*

Some curves obtained are given in Figs. 3*d* and 4. After the experiments it was found that the tension in the specimens in most cases had decreased almost completely during the heating. This was probably due to creep in the material. If the specimens were released after the exposures they contracted, within an hour, only a fraction of the original elongation.

It is seen that the final level of the curves in Figs. 3*d* and 4 is always higher than zero. This may be due to different reasons e.g. errors in the adjustment, a residue of oriented material, or, in the case of vulcanized specimens, disturbances from the recipe components. However, as only the temperatures at certain points of the curves were measured the position of the level at the end of the curves should not seriously affect the results.

With a few exceptions the curves show a marked decrease of crystallinity within a comparatively well defined temperature interval and in most cases some characteristic temperature values could be obtained. In Tables 3 and 4 some temperatures determined from the curves are given. The temperature at the first downward bend of the curve is denoted T_b and given as an interval. T_i is the temperature at the inflexion point of the step downward and T_m , the "melting temperature", means the intersection between the inflexion tangent and the linearly extrapolated last part of the curve. This melting point was somewhat lower (0.5–1°) than the temperature at the sharpest bend of the curve but the former value was preferred because it was more easily reproduced. Because of the comparatively large

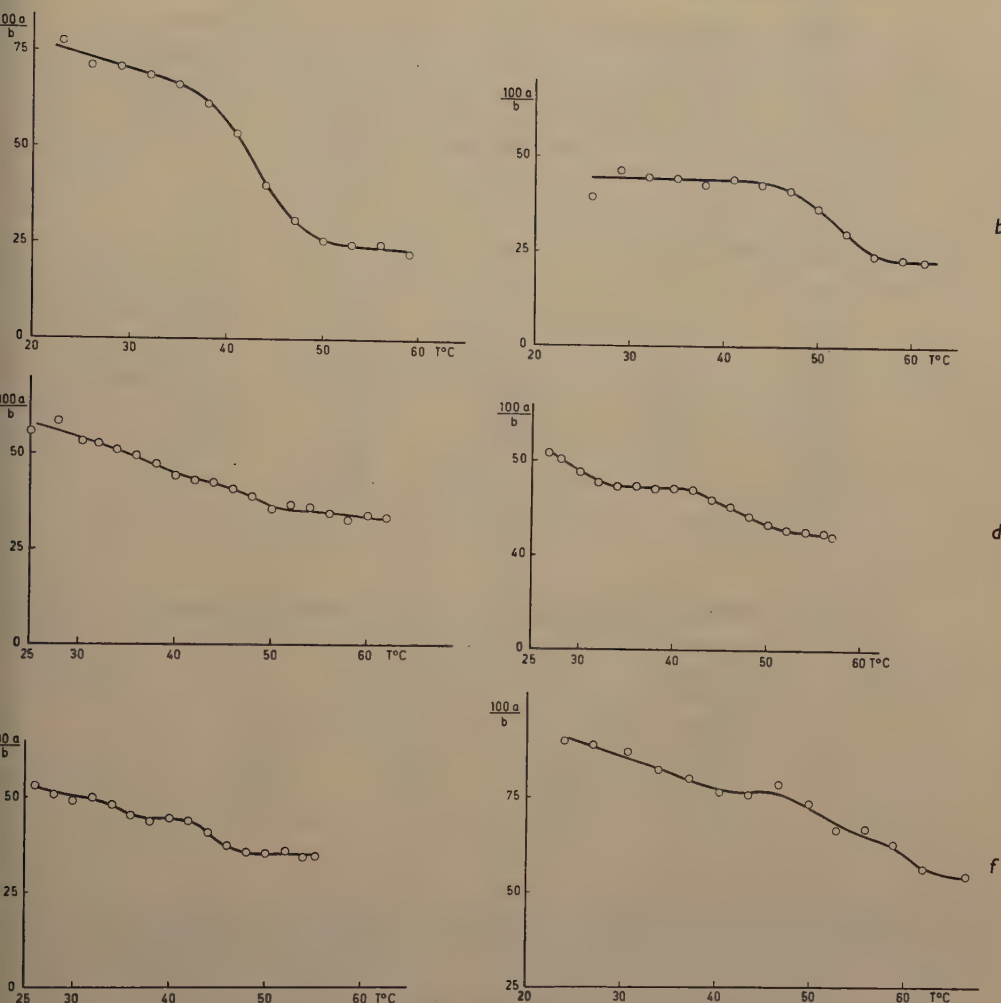


Fig. 4. Some curves obtained at constant deformation. (a) ANG unvulcanized; (b) E15 unvulcanized; (c) LB cured 20 minutes, average of two exposures; (d) LB cured 50 minutes, average of two exposures; (e) LB cured 60 minutes, average of three exposures; (f) ANE vulcanized (curing 45 minutes at 145°).

differences in the ordinate values for the different curves these values have not been considered here. In Table 3 are also given the intrinsic viscosities determined at this Institute in other investigations of neoprene (4, 5). Table 5 gives the measurements obtained from three different experiments at constant elongation (500 %; length of specimen $L_0(1 + 500/100)$; L_0 original length of specimen) with one material (LB vulcanized 15 minutes). The scattering of the measured values was in all the experiments about the same as in this case. The temperature value at which the curve bends off downwards is most uncertain; these values may differ by about 5°. The

Table 3. Experimental data for the unvulcanized specimens.

Intrinsic viscosities, $[\eta]$ in cm^3/g , measured in chloroform and temperature values determined from the curves (ordinates averaged if more than one experiment was made) obtained in the experiments at constant deformation.

T_b the temperature at the first downward bend of the curve
 T_i the temperature at the inflexion point of the downward step
 T_m the "melting temperature"

Material	$[\eta]$	T_b	T_i	T_m
ANE	93	33–35°	45.5°	48.5°
ANG	49	37–39°	43.5°	47.5°
E 15	324	44–46°	51.5°	56°
B 23 a	166	34–36°	41°	45.5°
Bö 7	113	32–34°	44°	49.5°
Bö 17	223	37–39°	47°	52.5°
Bö 22	126	32–34°	39°	44.5°
Bö 7 Bö 17	Specimens elongated 1000 % at +60° and stored at -4°.			{ 42° 40°

Table 4. "Melting points", T_m , for the vulcanized LB specimens.

Curing time minutes	T_m	Curing time minutes	T_m
10	35.5°	40	50.5°
15	46°	50	50.5°
20	50.5°	60	47°
30	53.5		

Table 5. Temperature values obtained in three experiments at constant deformation with vulcanized LB cured 15 minutes and elongated 500 %.

Notations the same as in Table 3.

Experiment	T_b	T_i	T_m
1	31–32°	40.5°	45.5°
2	34–36°	41.5°	46.5°
3	35–37°	43°	46°
Averages	33–35°	41.5°	46°

differences between the temperature values for the inflexion point could amount to about 3° and the temperatures at the intersection between the inflexion tangent and the extrapolated last part of the curve were usually reproduced within an interval of 2°.

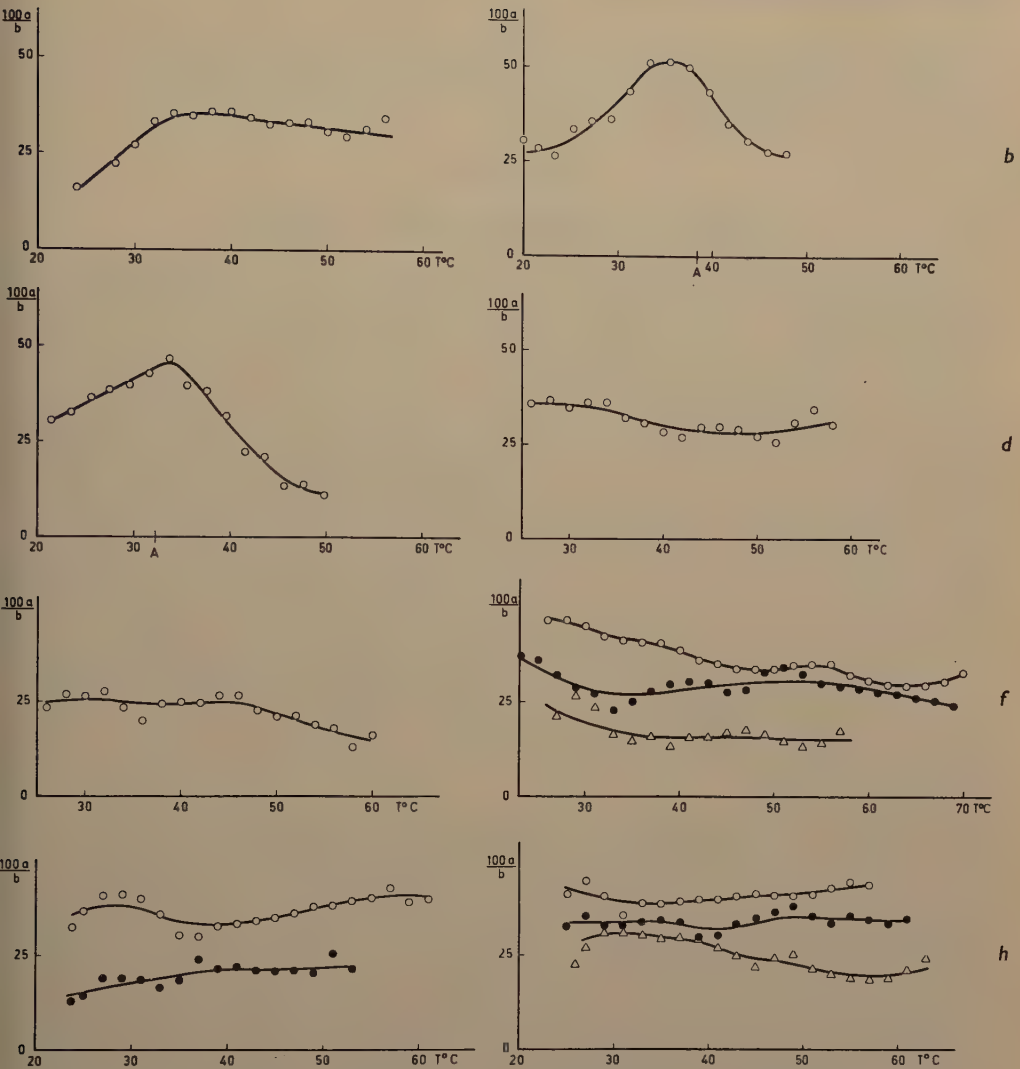


Fig. 5. Some curves obtained at constant load. *Unvulcanized specimens*: (a) ANE original elongation 10 %. (b) ANE original elongation 100 %. (c) ANE original elongation 250 %. (A in (b) and (c) marks the point where the specimens had reached the end of the specimen holder). *Vulcanized specimens*: (Notations cf. Table 2) (d) LB 10, original elongation 350 %. (e) LB 15, original elongation 300 %. (f) LB 20, original elongation $\triangle$ 100 %, $\bullet$ 250 %, $\circ$ 500 %. (g) LB 30, original elongation $\bullet$ 250 %, $\circ$ 400 %. (h) $\triangle$ LB 40, $\bullet$ LB 50, $\circ$ LB 60, original elongation 350 %.

(b) The experiments at constant load

As mentioned before the length of the specimen increased during these experiments. Initially this increase of length was slow but gradually became larger when the temperature was increased. The maximum extra elongation permitted by the specimen-holder varied, depending upon the starting conditions, but often it corresponded to an increase of the elongation of the specimen from 100 to 300 %.

The curves obtained in these experiments were more irregular than those found at constant deformation and it was not possible to determine characteristic values in the same way as in the latter experiments. Most of the curves obtained are given in Fig. 5.

Discussion

(a) Unvulcanized specimens at constant deformation

The ordinate values of the curves obtained depend upon the crystallinity in the specimens but it is also obvious that a change in the sharpness of the orientation will influence the values of the ordinates. However, before the experiments with continuous recording were started some experiments were made with the same thermostat camera in which a full X-ray diagram was recorded at different temperatures (B. Olofsson, unpublished experiments). On these diagrams no peripheral broadening of the maxima on the interference circle could be observed. These experiments were made with unvulcanized ANE at the elongations 300 %, 500 % and 700 %, and a value for the amount of crystallized material was determined from the diagrams. This value was different from the values used in this and the previous paper and only the temperature at which the crystallinity had completely disappeared could be compared with the values obtained in the experiments described in this paper. For the three elongations mentioned above, these temperatures were respectively 43°, 46° and 47°. The corresponding value for 500 % elongation obtained in the experiments described in this paper was found to be between 48° and 49° (Table 3) which may be reasonable agreement considering the difference between the methods. Because the temperature at which the crystallinity disappeared seemed to vary with the elongation most experiments at constant deformation were made at an elongation of 500 %.

The curves obtained in the experiments at constant deformation may be compared with melting curves although the process is not reversible. In some experiments the temperature was first increased to a value higher than the melting point and after that lowered again to about 30° but in these cases no recovery of the crystallinity was observed. It is well known that rubber and similar substances exhibit large hysteresis and time-dependence effects. When these and creep effects are taken into consideration it is evident that the experiments just mentioned were not complete but still they show that the process of melting is irreversible in this case.

Experiments in which the specimen had been kept at a fixed temperature until it had reached equilibrium might have taken considerable time. According to some dilatometric experiments by Flory, Mandelkern and Hall (6) on poly-(N, N'-sebacoyl-piperazine), equilibrium was not reached until after about 20 hours and in a crystallinity investigation on neoprene using infrared absorption spectra it was found by Mochel and Hall (7) that with some specimens crystallization continued for days after the stretching. The melting point obtained in experiments at a slow melting rate should be the ideal melting point (8) of the high polymer crystals. On the other hand melting points obtained in a fast melting process ought to depend both upon the experimental conditions and upon the state of the polymer. If conditions are kept constant, a criterion of which should be the reproducibility of the values obtained with different specimens of the same material, the values obtained should be more or less characteristic for the actual state of the polymer. In the following the term melting will be used for the process in these experiments and the temperature at

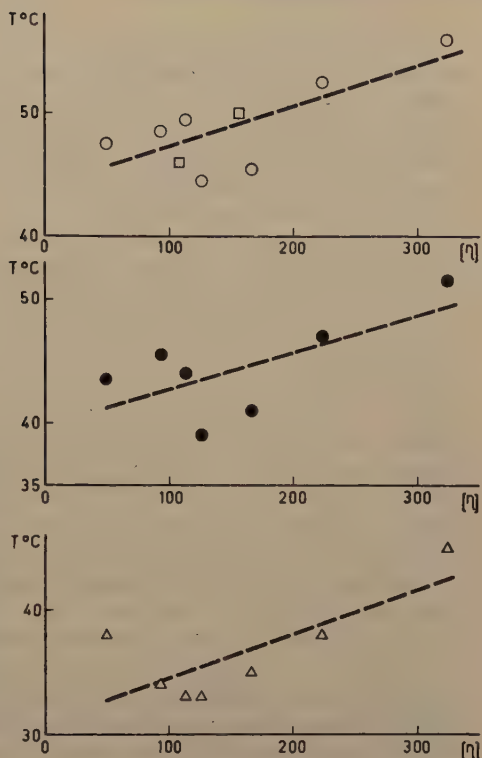


Fig. 6. Data derived from the curves obtained at constant deformation plotted against the intrinsic viscosities (in cm^3/g) of the materials in chloroform. Δ , the beginning of the melting, T_b . $\bullet$, the inflexion point, T_i . $\circ$, the melting point, T_m (the intersection between the inflexion tangent and the linearly extrapolated last part of the curve). The dashed lines are calculated from the experimental data according to the method of least squares. $\square$, melting points for materials polymerized at different temperature (from (10)) and intrinsic viscosities in benzene for the same types of polymers (from (12), (13)).

the intersection between the inflexion tangent and the linearly extrapolated last part of the curve will be called the "melting point", although it must be remembered that this is not a true thermodynamic melting point.

The specimens which were stretched at 60° and afterwards cooled and stored at -4° melted at lower temperatures than the same materials stretched at room temperature. The cold-stored specimens did not show the same well defined melting intervals as the others and the curves (an example in Fig. 3d) were somewhat like those obtained with vulcanized ANE (Fig. 4). In the experiments with the cold-stored specimens the exposures were started at 10 – 15° , the curves sloped downwards already from the beginning and the melting seemed to be finished at about 40° . A dependence of the position of the melting interval upon the crystallization temperature has been found in dilatometric experiments both for natural rubber (9) and for neoprene (10).

In Fig. 6 the values obtained from the melting curves for the unvulcanized specimens stretched at room temperature, i.e. T_b (the middle point of the interval), T_i and T_m are plotted against the intrinsic viscosities (in chloroform) for the specimens. The temperature values have been plotted against the intrinsic viscosities because the molecular weights were determined (from sedimentation and diffusion experiments (4)) only for four of the materials investigated here and also because the molecular weights probably were considerably more uncertain than the intrinsic viscosities. The general tendency in Fig. 6 seems to be that the temperature values increase with the intrinsic viscosities. It is seen that two of the values for T_m and

T_i lie considerably lower than the others whereas the points for T_b , which was least reproducible (cf. Table 5), lie on a curve with a minimum. From the data available it could not be decided whether the specimens giving the low T_m and T_i values were different from the others or not, and therefore the tendency of the experimental results has been marked (in Fig. 6) by the dashed lines, which are calculated from the points according to the method of least squares. It is seen that the lines are almost parallel.

A relation between the intrinsic viscosity, which is a property of the molecules in solution extrapolated to infinite dilution, and the melting properties for the crystallites (in bulk) in elongated specimens may seem peculiar. The differences between the melting temperatures are not very large but it seems improbable that a purely fortuitous distribution of the points would have given the picture shown in Fig. 6. It is well known (e.g. Flory's book (8)) that the intrinsic viscosities are related to the molecular weights and to the shape of the molecules in solution and it does not seem unlikely that these properties could also influence the rate of crystallization in elongated specimens (cf. (11)). In the present case, where the specimens were not given time enough to reach equilibrium it might be expected that the rate of crystallization would also influence the "melting points" determined.

In a paper by Maynard and Mochel (10) the melting points were determined with the dilatometric method for some, apparently unfractionated, neoprene materials polymerized at -10° , $+10^\circ$ and $+40^\circ$. The melting points obtained for specimens crystallized at room temperature were for the samples mentioned 67.5° , 50° and 46° respectively, the two last melting points being taken from the graphs in the paper (10). The authors mentioned also investigated the influence of different molecular weight for fractionated materials upon the crystallization and did not find any effect.

In the polymerization experiments in Uppsala the temperature was not kept constant; usually the reaction was started at about 25° to 30° and during the process the temperature was allowed to rise to about 40° . Probably the materials investigated by X-rays corresponded best to the $+40^\circ$ polymer in the paper by Maynard and Mochel. The value, 46° , for the melting point of this polymer is somewhat lower than most of the melting points found with the X-ray method (Table 3). Maynard and Mochel relate the differences in the melting points of their samples to the presence of polymerization products other than the *trans*-1,4 polymers. These other products (sections or links in the chains formed by *cis*-1,4 and by 1,2 and 3,4 polymerization) cause irregularities in the crystallites which decrease the melting point. It is believed that this relationship is not in contradiction to the result found in the experiments reported in this paper, but rather that the different relations give complementary aspects of the problem.

In earlier papers Mochel, Nichols and Mighton (12) and Mochel and Nichols (13) reported investigations of neoprene materials which were polymerized at $+40^\circ$ and $+10^\circ$ respectively. The materials were fractionated and intrinsic viscosities (in benzene) and molecular weights (osmotic) were determined both for the unfractionated polymers and for the fractions. It was found for the unfractionated materials that the $+10^\circ$ polymer had higher intrinsic viscosity and higher molecular weight than the $+40^\circ$ polymer. It is uncertain whether the same relation holds between the intrinsic viscosities for the materials investigated by Maynard and Mochel (10) but if it is so the higher intrinsic viscosity also in this case corresponds to a higher melting point. The values obtained from the papers (10, 12, 13) are for comparison also plotted in Fig. 6.

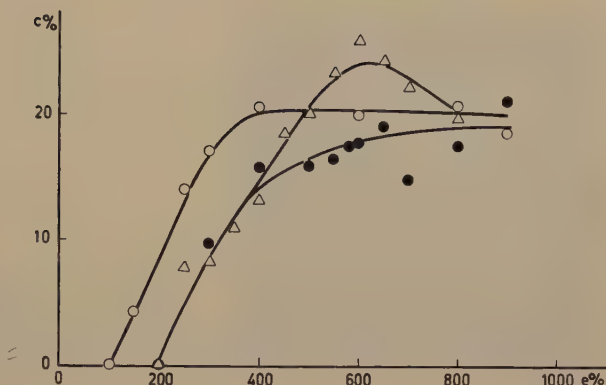


Fig. 7. Crystallinity, c , according to a previous paper (1) plotted against the elongation, e . ●, ANE unvulcanized; △, ANE vulcanized; ○, ANG unvulcanized.

(b) Relations between molecular and elongation-orientation properties

Considering the relation between the intrinsic viscosity and the data from the melting curves, it may also be mentioned that there seems to be a relation between the elongation-orientation curves, given in a previous paper (1) and the molecular properties for two of the materials (ANE and ANG) investigated. The other materials used in this investigation (1) were not quite normal, one being extremely hard and the other very soft and plastic, and do not fit into the following scheme. The elongation-orientation curves (from (1)) for ANE, vulcanized ANE and ANG are shown in Fig. 7. The elongation, e , is given in per cent of the original length, L_0 , of the specimen and thus the length of the specimen after stretching is $L_0(1 + e/100)$; c is a relative measure of orientation (crystallinity) based upon measurements of areas under the microphotometer curve taken along the interference circle (1). In case more than one exposure was taken at a given elongation only the average value of the orientation is plotted in the figure. It is seen that the orientation reaches a limiting (maximum) value at about 400 % and 600 % elongation for ANG and ANE respectively. The elongation beyond the value at which the limit of orientation was attained was probably for the unvulcanized materials due to creep in the specimens, and for the vulcanized sample accompanied by rupture of some bonds which caused a decrease of tension and of crystallinity. If it is assumed that the molecules in the oriented (crystallized) part of the materials have reached the maximum extension at the limiting value of orientation, it can be estimated from the curves in Fig. 7 how much the different materials had to be stretched to attain such a state. The elongation values above show that this extension was reached for ANG when it had been stretched to five times its original length and for ANE at seven times the original length.

For neoprene materials the degree of crystallization should depend, among other things, upon the degree of extension of the molecules and for similar materials the corresponding relative values of crystallinity and of extension ought to be the same. In this case a measure of the extension may be taken as the quotient between the actual length of the specimen and the length when the maximum crystallinity was attained. Thus for ANG, for which this state seemed to be reached when the specimen had been stretched to five times its original length, the extension value is 0.40 at the elongation of 100 %, where crystallinity was first observed. If the elongations in Fig. 7 are recalculated in extension values the curves shown in Fig. 8 are obtained.

It is seen that the first parts of the curves practically coincide indicating similarity in the dependence of crystallinity upon extension for the different materials. The deviations from the common part of the curve may be due to creep effects or to prevention of such because of vulcani-

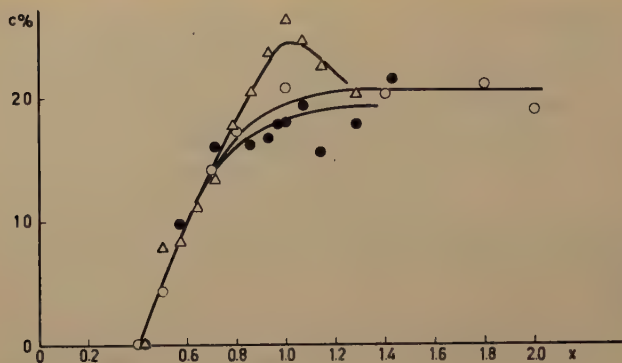


Fig. 8. The crystallinity values from Fig. 7 plotted against the extension values, x , according to the text. ●, ANE unvulcanized; △, ANE vulcanized; ○, ANG unvulcanized.

zation. It is also possible to plot the value for the crystallinity in relation to the maximum crystallinity for the different materials but in this special case the difference between the two ways of plotting is not very large.

The length of the specimen may also be given in relation to the length of the unstretched specimen. If these values for the lengths are compared, it is seen that the quotient between the lengths of the stretched unvulcanized specimens of ANE and ANG, at the extensions where the crystallinity was first observed, is 1.5 and the same quotient when the limiting value of crystallinity was attained is 1.4. It may be noted that these values are not very different from the cube root of the quotient between the molecular weights of the specimens (61,000 and 204,000 for ANG and ANE respectively (4); $(\frac{204}{61})^{\frac{1}{3}} = 1.50$), but as the molecular weights are uncertain this relation might be accidental.

The elongation values at the beginning of crystallization also give an aspect of the elastic behaviour of materials with different molecular weights. If crystallization begins at lower elongations for low molecular weights than for higher ones, the region of ideal rubber elasticity should be more limited for low than for high molecular weights.

In the above discussion it has been possible to compare only two different unvulcanized materials. The elongation-crystallinity curves depend upon the experimental conditions (1) and the materials investigated may have had not only different molecular weights but also some differences in structure. Still the result indicates the existence of relations between properties of the materials in bulk and their molecular properties.

(c) *Vulcanized specimens at constant deformation*

In most cases (Fig. 4) a melting point could be determined, but it was not possible to compare the melting points for unvulcanized and vulcanized samples of the same polymer, because the unvulcanized sample of the material (LB) which gave melting points in the vulcanized state had been masticated and gave only a very weak trace of crystallinity when it was stretched 500 %. On the other hand the material ANE which could be crystallized in the unvulcanized state and which also was investigated after vulcanization did not give a well defined melting point in the vulcanized state (cf. (2)).

As with the masticated LB material weak crystallization was obtained with the masticated and mixed but uncured material. In the last case the interferences from the recipe components showed marked orientation effects. An exposure is given in

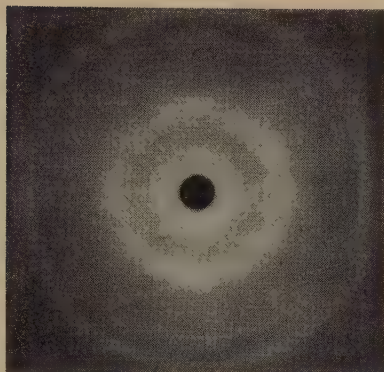


Fig. 9. X-ray diagram from a specimen of masticated and mixed LB material elongated 500 %. Orientation effects are clearly observed on the interference circles from the recipe components whereas only a very weak effect is seen on the interference circle from the polymer.

Fig. 9. With the material cured 5 minutes crystal interferences were obtained from the polymer but the melting point could not be determined, possibly because the specimen melted at a low temperature. The specimen cured 10 minutes crystallized and melted; the melting started at a lower temperature than 20° and was completed at about 36° . With the next specimen in the series, cured 15 minutes, a curve was obtained which closely resembled the curves from the experiments with unvulcanized specimens but the following vulcanized specimens showed some deviations. Somewhere between 35° and 45° the curves for the specimens cured 20, 30 and 40 minutes showed a tendency to bend off horizontally and for the specimens cured 50 and 60 minutes it was quite obvious that there was a horizontal plateau with a length of several degrees. After that, the curves bent downwards to the melting point. Vulcanized ANE, for which no definite melting point could be obtained, showed signs of horizontal parts although not very marked and when the temperature was increased in order to reach a region where the curve remained at constant level the specimen broke at about 65° to 70° . The same result was also obtained with a specimen elongated 400 %.

The irregularities in the melting curves for the vulcanized materials may be compared with some effects found in melting experiments with vulcanized natural rubber at constant deformation (e.g. (14)). In the present case it may be expected that the vulcanized specimens contained a certain amount of imperfect crystallites where irregularities were caused by the cross-linking. The melting of this part of the material probably started immediately when the specimen was heated and caused the slope, often found at the first part of the curve. The remaining crystallites ought to have higher melting points and it might be expected that the melting process would stop temporarily. Of course there may also be simultaneous processes of melting and recrystallization which counterbalance each other under certain conditions. As it was generally observed that the tension in the specimen had decreased during the heating it seems that there had also been some creep. If the cross-linkages did not essentially prevent this effect during the early stages of the experiment it seems likely that the melting would have continued as with the unvulcanized specimens. It is very probable that the plastic deformations were connected mainly with the last part of the experiments as it was observed that at constant load the rate of elongation was slow at the beginning of the heating. Of course, the time dependence

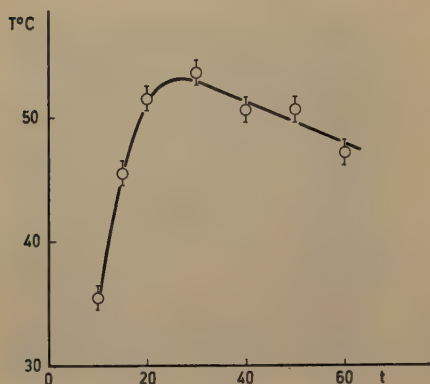


Fig. 10. The melting temperatures for the vulcanized LB specimens plotted against the curing time, t , in minutes.

usually found in high-polymer reactions may also have had some influence upon the shape of the melting curves but it can hardly have been the only factor involved.

If the melting points for the series of LB vulcanizates are plotted against the curing time a curve with a maximum is obtained which is shown in Fig. 10. This curve may be compared with the two curves obtained by Field (2) for the amount of crystalline material at constant elongation against the curing time for vulcanized specimens of natural rubber. Field's curves also increase rapidly to a maximum and beyond that decrease slowly. In all cases the explanation may be the same; at low degrees of vulcanization the crystallization is favoured (creep prevented), compared with the masticated material, but a high degree of cross-linking disturbs the crystallization to a certain extent.

(d) *The experiments at constant load*

In these experiments it was difficult to work with unvulcanized materials because the specimens broke or within a comparatively short time had been elongated as much as the specimen holder permitted (i.e. two or three times the elongation when the experiment was started). When the specimens had been stretched to the bottom of the specimen holder the exposure continued under conditions of constant deformation. However, results of some interest were obtained with unvulcanized ANE. This material had been stored for at least one and perhaps two or three years during which time it probably had crystallized to a certain extent. The preparation of the specimens probably did not completely destroy the crystallites already formed and ANE required a higher load than other unvulcanized materials to reach a certain elongation. Crystallites present in the unstretched material were randomly oriented and did not influence the value determined for the amount of oriented material. Experiments with unvulcanized ANE were made with three different loads which were adjusted to give elongations of 10 %, 100 % and 250 % (Table 2a). The corresponding curves are given in Fig. 5a, b, c. It is seen that in all three cases the amount of oriented crystallites first increases, probably due to a reorganization of the material; during the heating the specimen softened because of the melting of crystallites present before the application of the load, then the specimen was elongated further and oriented crystallites were formed which caused the observed value of the crystallinity to increase. With the smallest load the crystallinity increased to a

temperature of about 34° and after that remained almost constant. In this case the specimen had not been elongated as much as the specimen holder permitted when the exposure was interrupted at about 56° . With the second load the crystallinity also increased to about 34° where the curve seemed to bend off horizontally but in this case the limit of the specimen holder was reached at 38.5° after which the curve bent downwards as in the experiments at constant deformation and showed a melting point at about 46° . At the largest load, with the original elongation 250 %, the crystallinity also passed a maximum but in this case the limit of the specimen holder was reached at 32° and shortly afterwards the curve bent downwards without indication of a horizontal plateau as in the second case. In the last experiment the melting point also was about 46° .

The vulcanized specimens investigated at constant load belonged to the LB series of vulcanizates (Table 2*b*). These curves are shown in Fig. 5*d-h*. In all the cases for which the curves are given, the elongation of the specimen never reached the limit set by the specimen holder. The curves are rather irregular, which may partly be due to the method but probably also depends upon the specimens (differences in the area of the cross section etc.) and upon the process recorded. The crystallinity in the specimens seems to depend upon the original elongation (Fig. 5*f, g*), no melting points can be obtained from the curves and no definite relation between the curing time and the levels of the curves can be found.

In these experiments with vulcanized materials the specimens were elongated by the load during the exposure, which was somewhat unexpected. The elongation could not be measured for reasons discussed in the section dealing with the method. The deviation from what would be expected according to the theory of rubber elasticity is probably due to the fact that the elongation was so large that the conditions for ideal rubber elasticity were no longer fulfilled. If it is assumed that the cross-linkages formed at the vulcanization essentially prevented creep in the material, at least during part of the heating, there seems to have been a continuous and complex process of melting, chain extension and recrystallization in such a way that the amount of oriented material obtained at the original elongation was mainly preserved. A similar explanation, supplemented for creep effects, would also fit the result from the experiments with unvulcanized ANE, although for this material there was a definite "running in" period, probably due to the presence of unoriented crystallites.

Considering the irregularities in the curves obtained with the vulcanized specimens at constant deformation and the fact that no melting points were obtained in the experiments at constant load, it seems likely that the melting points obtained at constant deformation depend upon reciprocal action between the melting process and the decrease of tension (creep) observed in these experiments. In that case the agreement between the dilatometric (10) and X-ray melting points may be natural.

Summary

A method is described for the continuous recording of X-ray interferences from an elongated specimen while it is heated. By this method relative values of the crystallinity (orientation) for elongated neoprene specimens can be obtained as a function of the temperature of the specimen. Materials were investigated both at constant deformation and at constant load.

In the experiments at constant elongation a "melting point" was usually obtained and it was also found that the tension of the material in most cases decreased markedly

during the heating. For the unvulcanized specimens "melting points" (Table 3) between 45° and 56°C were found and these seemed to increase with the intrinsic viscosities for the materials in solution.

Relations between the elongation-crystallinity curves (from (1)) and the molecular properties for two of the materials investigated here have been discussed.

For a series of vulcanizates of the same composition but with different curing times, "melting points" at constant deformation were also obtained. These seemed to be in relation to the curing times. Those specimens which had been cured for about the normal time (45 minutes) gave "melting points" about 50°C. The melting curves for the vulcanized specimens at constant deformation in some cases went down in steps which were not found for the unvulcanized specimens.

In the experiments at constant load the specimens were elongated by the load when the temperature was increased during the exposure. The crystallinity did not change much during the heating and no "melting points" were obtained.

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Orientation and hydrogen isotope effect in nitration of anisole

By KJELL HALVARSON and LARS MELANDER

With 2 figures in the text

It has been known for a long time that the orientation in the nitration of anisole is strongly dependent on the conditions of nitration [1]. Reagents of the acyl nitrate type seem to give a much higher proportion of the ortho derivative than ordinary nitration media in which the nitronium ion is the most probable attacking entity. The isomeric proportions have earlier been determined by means of melting point methods, at least in the case of the ortho/para ratio. Since infra-red spectrophotometry offers a simple and fairly accurate method of determining these proportions, it has been considered of interest to corroborate the earlier results by this independent method.

During the work, the possibility of generating the acyl nitrate *in situ*, as done by Burton and Praill [2], was considered. The use of acetonitrile as solvent, as recommended by these writers, caused further increase in the ortho/para ratio, as compared to that obtained by Griffiths, Walkey, and Watson [1].

The difference in orientation between the various media could be ascribed to different attacking entities being operative, e.g. dinitrogen pentoxide [3] and the nitronium ion [4]. Under some conditions the nitrosonium ion might also act as substituting agent [5]. In the case of the nitronium ion, the hydrogen replacement reaction is known to proceed without any observable kinetic hydrogen isotope effect [6, 7, 8]. If the mechanism of hydrogen replacement is different for the other agents, it might be worth while to investigate whether an isotope effect arises. A concerted mechanism has been considered for dinitrogen pentoxide [9], and, if such a mechanism is realized, an appreciable isotope effect should arise.

Tritium was used for the study of the isotope effect. It may be used in tracer amounts and will accordingly not influence the observable infra-red spectrum of the product.

Three different types of nitration were performed: with nitric acid in acetic anhydride, with benzoyl nitrate in carbon tetrachloride, and with benzoyl chloride and silver nitrate as nitrating agent in a solution of acetonitrile. Isotope effect measurements were made in the last type of reaction.

For comparison, some experiments were also carried out with toluene. Two kinds of nitrations were performed, one with benzoyl nitrate in carbon tetrachloride and the other with benzoyl chloride and silver nitrate in acetonitrile. In the latter case some isotope effect measurements were made.

Experimental

Tritium.—The tritium used originates from the Atomic Energy Research Establishment, Harwell.

Determination of tritium.—The combustion of anisole-2-*t* and toluene-2-*t* was carried out over Vinosit B at 650°C in a stream of oxygen. The nitro compounds were burned in a stream of carbon dioxide at 650°C over Vinosit B, after which the combustion products were passed through a second furnace containing pieces of reduced and heated copper wire. The tritium in the water obtained from the combustion was determined by inside counting in a Geiger-Müller tube, according to the method described earlier [6, 10]. The accuracy in these determinations is $\pm 2\%$. When corrections for the natural decay of tritium were needed, its half-life was assumed to be 12.5 years.

Experiments with anisole

Preparation of tracer amounts of anisole-2-t.—The anisole-2-*t* was prepared in a Grignard reaction in the ordinary manner. *o*-Bromoanisole (T. Schuchardt) was distilled under reduced pressure in a Vigreux column and the fraction boiling at 110.0–110.4°C at constant pressure (about 20 mm), was collected. A three-necked flask was equipped with a stirrer, a condenser and a dropping funnel with a drying tube. 3.4 g (0.14 mol) of magnesium turnings and 10 ml of a solution of 25.7 g (0.137 mol) of *o*-bromoanisole in 25 ml of ether were placed in the flask. Some very small pieces of iodine were added and the reaction then started after about 20 minutes. 25 ml of ether was added to the reaction mixture and another 25 ml to the rest of the *o*-bromoanisole-ether solution, which was placed in the dropping funnel and added drop by drop. The reaction proceeded for two hours after which the mixture was gently boiled for half an hour. The Grignard solution was cooled in ice water, 2 ml of anisole was added and the radioactive water (200 mm³ or less) then added drop by drop with frequent stirring. The reaction mixture was left for two days with intermittent stirring and finally 10 ml of anisole and 10 ml of water were added. The solution was filtered off and the remaining magnesium salts were washed with a little anisole, which was combined with the filtrate. The solution was shaken with water, dried with Drierite, and then distilled under reduced pressure in a Vigreux column. The fraction boiling at 50.4–50.7°C at constant pressure (about 15 mm) was collected. Two batches of anisole-2-*t* were prepared having an activity yield of 10 % and 26 %; the theoretical yield is 50 % if there is no isotope effect.

Nitration with nitric acid in acetic anhydride.—To be able to compare the experimental values of Griffiths *et al.* [1] with the present ones, one of their experiments was repeated. In a double-walled thermostated flask, 6.3 g of nitric acid ($d = 1.5$) was diluted to a total volume of 63 ml with acetic anhydride and the solution cooled to +10°C. 10 g of anisole was added and the mixture was shaken for one hour at +10°C. The reaction mixture was poured into icewater and the solution made slightly alkaline with sodium hydroxide solution. The nitroanisole was separated off, washed with a little water, dried with Drierite and distilled under reduced pressure in a short Vigreux column. The material boiling at 142–46°C, 15.5 mm, was collected and the residue then consisted of the hold up (0.3 g). Yield 81 %. The isomeric composition in the product is given in Table 1, Exp. 1.

Table 1. Nitration of anisole

Experiment	1	2	3	4	5	6	7	8
Temperature, °C :	10	0	0	0	0	0	0	0
Mol ratio*	—	—	1:1.8	1:1.25	1:1.03	1:1.8	1:1.8	1:1.5
Time of reaction, h	1	3	3	4	4	5	6.5	7
Yield, %	81	58	24	28	37	66	75	81
<i>o</i> -Nitroanisole, % of yield	71	71	76	75	74	75	74	75
<i>m</i> -Nitroanisole, % of yield	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
<i>p</i> -Nitroanisole, % of yield	28	29	24	25	26	25	26	25

* Mol ratio between anisole and nitrating agent.

Nitration with benzoyl nitrate in carbon tetrachloride [2, 3, 11].—A three-necked flask was equipped with a two-necked adapter to which was attached a stirrer and a drying tube containing phosphorus pentoxide. One of the side necks was joined with a bent tube to a bottle containing powdered silver nitrate. The other side neck also had a joint, containing a fairly large sintered glass disk, and ending in a suction flask equipped with a drying tube on the side arm. In the former flask 48 g (0.34 mol) of freshly distilled benzoyl chloride and 70 ml of carbon tetrachloride were placed. The solution was cooled to -15°C in a bath of dry-ice and alcohol. The silver nitrate (73 g, 0.43 mol) had been fused, ground and dried under reduced pressure at 120°C , and the reaction was started by gentle rotation of the bent tube. When all the silver nitrate had been added (3 hours), the mixture was stirred for another hour. The solution was then sucked off through the glass filter into the cooled (-10°C) suction flask. The solution was stored at -10°C in a flask placed in a desiccator.

The nitration with the prepared benzoyl nitrate was carried out in the following manner. A 250 ml three-necked flask was equipped with a stirrer, a thermometer with long stem and a dropping funnel with a drying tube. 10 g of anisole and 50 ml of carbon tetrachloride were placed in the flask. The solution was cooled to 0°C and 45 ml of the benzoyl nitrate solution was added through the dropping funnel as rapidly as possible with frequent stirring and maintaining the temperature at 0°C . The amount of benzoyl nitrate added was assumed to be equivalent or in excess, compared with the amount of anisole, but the benzoyl nitrate solution was not analyzed. The total time of reaction was three hours and the reaction mixture was then poured onto crushed ice. Dilute sodium hydroxide solution was added and the solution was shaken intermittently and then left overnight. The carbon tetrachloride was separated off from the alkaline solution, dried with Drierite and the solvent distilled off. The remaining nitroanisole was distilled under reduced pressure in a short Vigreux column. The material boiling at $135\text{--}39^{\circ}\text{C}$, 11.5 mm, was collected and the residue consisted of the hold up (0.3 g). Yield 58%. The composition of isomers in the product is given in Table 1, Exp. 2.

Nitration with benzoyl chloride and silver nitrate in acetonitrile.—Six nitrations were carried out with anisole-2-*t*. The nitrating agent was benzoyl chloride and silver nitrate in a solution of acetonitrile [2]. The reactions were carried out in a three-necked 500 ml flask equipped with a stirrer, a dropping funnel with a drying tube, a thermometer with long stem, and a condenser with a drying tube. 69.7 g (0.41 mol) of ground and dried silver nitrate was dissolved in 200 ml of acetonitrile and then

cooled to 0°C in a mixture of ice and salt, after which 24.9 g (0.23 mol) of anisole-2-*t* was added. The benzoyl chloride, which was mixed with 80 ml of acetonitrile, was added cautiously through the dropping funnel. The amount of benzoyl chloride varied between 48.6–58.3 g in the different experiments, which is equivalent to 0.35–0.41 mol. The above figures are valid for Exp. 6–8; in Exp. 3–5 half quantities were taken except for the benzoyl chloride of which 16.7–29.2 g was added, which equals 0.119–0.208 mol. The time of addition of the benzoyl chloride solution varied from 30 to 60 minutes and the reaction mixture, the temperature of which was held at 0°C, was stirred rapidly. About half an hour after the beginning of the reaction, the white mixture became yellow. The time of reaction varied from 3 to 7 hours. The mixture was poured onto crushed ice and water containing sodium chloride, the purpose of which was to react with the unconsumed silver nitrate. About 20 ml of benzene was added and the whole mixture filtered with suction, after which the silver chloride was washed with a little benzene. Dilute sodium hydroxide was added to the filtered solution and the mixture was shaken and left overnight. The alkaline water phase was separated off from the organic phase, which was dried with Drierite. The benzene was distilled off at ordinary pressure, the unreacted anisole-2-*t* under reduced pressure (about 50°C and 15 mm), and finally the remaining nitroanisole was distilled off (110–115°C and 3–4 mm). The small residue in the distillation flask consisted of the hold up, which was liquid even after cooling, indicating that little or no dinitrated products were formed during the reaction [5]. The recovered unreacted anisole-2-*t* was redistilled in a small Vigreux column and the fraction boiling at 51.3–51.5°C at constant pressure (about 16–17 mm) was taken. The isomer composition of the different samples is given in Table 1, Exp. 3–8.

Exchange experiments.—The hydrogen atoms in the anisole ring are easily exchanged in a strongly acid medium. Before the experiments with anisole-2-*t* were started, two reactions were carried out in order to check if any hydrogen exchange occurs. The reactions were carried out in the same manner as that used in the nitrations of anisole with benzoyl chloride and silver nitrate, but inactive anisole was used and a small amount of tritiated water (5 mm³) was added to the reaction mixture. In the first experiment the water added should give a specific activity of 200 (in the empirical unit used) calculated on the assumption of a random distribution of isotopic hydrogen throughout the reaction mixture; a sample of unreacted recovered anisole gave a specific activity of 0.2. In the second experiment, the corresponding activities were 25 and 0.05 respectively; in this case the activity was measured on the nitroanisoles formed.

Check of the behaviour of the nitroanisoles during the working up.—In order to check if there is any appreciable change in the isomeric composition during the working up and the distillation, a known mixture of nitroanisoles was treated in the same way as the nitroanisoles obtained by nitration of anisole-2-*t*. The nitroanisoles were dissolved in acetonitrile, and benzoic acid and benzoic anhydride were added to the solution. The solution was then stirred for one hour. The mixture was poured into water containing a little sodium chloride, some benzene was added and the mixture shaken well. Dilute alkali was added in slight excess and the whole solution intermittently shaken and left overnight. The organic phase, containing the nitroanisoles, was worked up and distilled under reduced pressure, all as in the nitration experiments. Two samples were taken and the isomer composition determined: (1) original weighed mixture of nitroanisoles, ortho 75.2 %, meta 3.0 %, para 21.8 %; (2) mixture worked up as above, ortho 75.3 %, meta 3.0 %, para 21.7 %. These figures show that

there is no significant change in isomeric composition during the working up of the material. The concentration of *m*-nitroanisole was chosen as high as 3 % because the actual concentration in the nitration experiments was too low to be measured with any accuracy.

Experiments with toluene

Preparation of tracer amounts of toluene-2-t.—The toluene-2-*t* used in these experiments was prepared by means of a Grignard reaction.¹ The starting material was 2-bromotoluene (T. Schuchardt) and the reaction was carried out as described earlier [6]. The boiling point of the toluene-2-*t* used was 109.6–110.0°C.

Nitration with benzoyl nitrate in carbon tetrachloride.—The experiment was carried out in the same manner and with the corresponding amounts of material as described for anisole. The nitrotoluenes had a boiling point of 108–114°C at 16 mm. The yield was 30 % and the isomeric composition of the product is given in Table 2, Exp. 9. Less volatile products had been formed in an amount not exceeding 4 %.

Table 2. Nitration of toluene

Experiment	9	10	11
Temperature, °C	0	0	0
Mol ratio*	—	1:1.02	1:2.4
Time of reaction, h	3	3	4
Yield, %	30	32	41
<i>o</i> -Nitrotoluene, % of yield . . .	57	59	57
<i>m</i> -Nitrotoluene, % of yield . . .	6	5	4
<i>p</i> -Nitrotoluene, % of yield . . .	37	37	38

* Mol ratio between toluene and nitrating agent.

Nitration with benzoyl chloride and silver nitrate in acetonitrile.—Two experiments were carried out and the following amounts of material were used in the first one: 18.7 g (0.11 mol) of silver nitrate was dissolved in 35 ml of acetonitrile and then 9.95 g (0.108 mol) of toluene-2-*t* was added. A solution of 15.5 g (0.11 mol) of benzoyl chloride in 15 ml of acetonitrile was added drop by drop to the cooled (0°C) solution of silver nitrate, toluene-2-*t* and acetonitrile. Boiling point of nitrotoluenes, 104–110°C at 12 mm. The total time of reaction was 3 hours and the yield 32 %. In the second experiment (Exp. 11), the amounts of material were: 156 g (0.92 mol) of silver nitrate, 400 ml of acetonitrile, 35 g (0.38 mol) of toluene-2-*t* and 129 g (0.92 mol) of benzoyl chloride mixed with 165 ml of acetonitrile. Boiling point of nitrotoluenes, 106–112°C at 14 mm. The total time of reaction was 4 hours and the yield was 41 %. The isomeric composition is given in Table 2, Exp. 10–11. Less volatile products were formed in an amount not exceeding 4 %. The recovered unreacted toluene-2-*t* was distilled in a small Widmer column. Boiling point 109.0–109.3°C (Exp. 10) and 109.2–109.5°C (Exp. 11).

¹ The toluene-2-*t* was prepared by Fil. kand. Stig Olsson at this institute, who very kindly placed it at our disposal.

Check of the behaviour of the nitrotoluenes during the working up.—The nitrotoluenes were distilled twice, contrary to the nitroanisoles, which were distilled only once and then used for measurements. The distillation was not forced as far as in the case of the nitroanisoles and 10 % (by volume) of the material was left as residue in the first distillation. This procedure caused an appreciable change in the isomeric composition and with the column used, the ortho isomer increased with 7 relative per cent while the meta and para decreased with 10 and 14 relative per cent, respectively, in the distillate. Corresponding corrections were applied to the results. An experiment was made in a manner corresponding to that with the nitroanisoles to check the other stages of the working up, but there was only a minute change in isomer composition, just as in the earlier case.

Determination of isomeric composition

Infra-red determination of o-, m-, p-nitroanisoles and o-, m-, p-nitrotoluenes. The infra-red determinations were performed using a Perkin-Elmer spectrophotometer Model 112.¹ The solvents used, carbon disulphide for the nitroanisoles and carbon tetrachloride for the nitrotoluenes, could be considered as perfectly transparent at the wavelengths concerned. It was consequently possible to treat the mixtures as three-component systems.

The wavelengths chosen were, for the nitroanisoles 13.44μ (744 cm^{-1}), 12.47μ (802 cm^{-1}), and 11.84μ (845 cm^{-1}) (slit widths used 0.700, 0.500, and 0.400 mm, respectively) and for the nitrotoluenes 8.68μ (1152 cm^{-1}), 9.11μ (1098 cm^{-1}), and 8.99μ (1112 cm^{-1}) (slit width for all three 0.200 mm) where the o-, m-, and p-derivatives, respectively, have absorption maxima. Standard curves were made up for each one of the three wavelengths. Two matched 0.11 mm sodium chloride cells were used, one for the solvent and one for the solution. Beer's law was obeyed with sufficient accuracy to give constant extinction coefficients. The composition of the unknown samples could consequently be calculated from the absorption measurements by means of three linear equations. Pristera and Halik [12], who made infra-red analyses on o-, m-, and p-nitrotoluene and 2,4-dinitrotoluene used the method of successive approximations in determining the isomer composition. In their case it seems to have been quite laborious to use the present, more direct method as they have a five-component system including the solvent.

In order to check the analytical method, artificial mixtures of compositions similar to those of the unknown samples were made. Minor deviations from ideal behaviour caused errors of 2 to 6 relative per cent for the different isomers, these errors being fairly constant within the intervals studied. Corresponding corrections were applied to the results. The final results are believed to be accurate within $\pm 2\%$ for o- and p-nitroanisole and $\pm 3\%$ for o- and p-nitrotoluene. For m-nitrotoluene, being present in lower concentrations, the relative error was $\pm 10\%$, and for m-nitroanisole no finite amount at all could be observed in the unknown mixtures. In the latter case an actual percentage of 0.5 would have been observable.

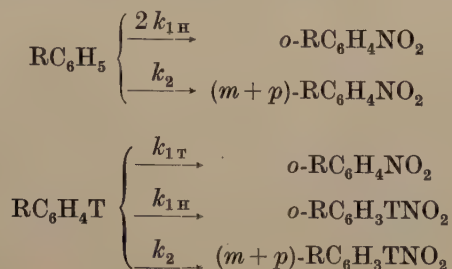
Calculations and results

Calculations used earlier for the computation of the kinetic isotope effect in aromatic substitution [6] could, with minor modifications, be applied to the present

¹ We wish to thank Fil. mag. Ulla Berglund-Larsson for valuable advice in handling the spectrometer.

problem. In the present experiments, a mixture of at least two isomers of the mono-derivative results, and the tritium has been determined without isomer separation. The isotope effect in the nitration of the ortho position has been studied, since the ortho derivative is the predominant one.

The following reaction scheme is valid, provided the isotopic hydrogen influences only the specific reaction rate of its own position:



The specific rates have been given above the arrows; index 1 refers to substitution in a single ortho position, and index 2 to the combined substitutions in the meta and para positions. All tritium organically bound is located ortho to the original substituent R.

If i denotes the concentration of ordinary molecules and a that of the tritiated ones, the following expression applies:

$$\frac{-da}{-di} = \frac{k_{1\text{T}} + k_{1\text{H}} + k_2}{2k_{1\text{H}} + k_2} \cdot \frac{a}{i} = \frac{\beta + 1 + q}{2 + q} \cdot \frac{a}{i},$$

where $\beta = k_{1\text{T}}/k_{1\text{H}}$ and $q = k_2/k_{1\text{H}}$. This expression holds only if the reactions are of the first order with respect to the aromatic molecule. Integration gives

$$\frac{a}{a_0} = \left(\frac{i}{i_0} \right)^{\frac{\beta+1+q}{2+q}} = (1-x)^{\frac{\beta+1+q}{2+q}},$$

where the index 0 denotes initial concentrations and the fraction having reacted, $x = (i_0 - i)/i_0$, has been introduced.

The specific activity of the remaining anisole relative to that of the initial one may, for tracer amounts, be represented by

$$g(x, \beta) = \frac{a/i}{a_0/i_0} = \left(\frac{i}{i_0} \right)^{\frac{\beta-1}{2+q}} = (1-x)^{\frac{\beta-1}{2+q}}$$

or

$$\log g(x, \beta) = \frac{1-\beta}{2+q} [-\log(1-x)].$$

Of the tritium originally present, the fraction

$$\frac{k_{1\text{H}} + k_2}{k_{1\text{T}} + k_{1\text{H}} + k_2} = \frac{1+q}{\beta+1+q}$$

will be found in the mixture of substitution isomers. The specific activity of the latter relative to that of the initial material will consequently be

$$f(x, \beta) = \frac{\frac{1+q}{\beta+1+q} \cdot \frac{a_0 - a}{i_0 - i}}{\frac{a_0}{i_0}} = \frac{1+q}{\beta+1+q} \cdot \frac{1}{x} \left[1 - (1-x)^{\frac{\beta+1+q}{2+q}} \right].$$

The fraction of ortho derivative in the mixture of isomers is $2k_{1H}/(2k_{1H} + k_2) = 2/(2+q)$, because the tritium-containing tracer amounts may be neglected in this connection. This fraction, and hence q has been determined from infra-red spectrometry. This in principle allows β to be calculated from specific activity measurements on the initial material and either the remaining part of the latter or the resulting mixture. Thus no separation of isomers will be necessary.

The function $f(x, \beta)$ is the same as that of equation (9:9) of reference [6], only with $n = q + 2$.

In the nitration of anisole with benzoyl chloride and silver nitrate in acetonitrile, the percentage of the ortho isomer was found to be close to 75 (Table 1), thus

$$\frac{2}{2+q} = 0.75,$$

$$q = \frac{2}{3}.$$

In Figs. 1 and 2 the experimental values of the isotope effect determinations have been compared with the theoretical functions $f(x, \beta)$ and $g(x, \beta)$ for certain values of β . The deviations from the horizontal lines corresponding to $\beta = 1$ are about what is to be expected from the accuracy of the tritium determination. According to Fig. 2, $\beta = 1.00 \pm 0.03$. There is some risk, however, that the benzoyl nitrate forms so rapidly and is so reactive that the reaction is completed before the nitrating reagent has been uniformly distributed in the mixture. In such a case there is no fair competition between anisole and anisole-*t*, as the concentrations do not have sufficient time to equilibrate throughout the mixture. The reaction is then forced to consume an anomalous amount of the tritium. This would cause the experimental points to approach the line for $\beta = 1$ in Fig. 2, but would have the opposite effect in Fig. 1. It seems safe, then, to give the result as $\beta = 1.0 \pm 0.1$, especially as the incompleteness of the reaction and other observations give little justification to the fear.

In the two experiments on the nitration of toluene with benzoyl chloride and silver nitrate in acetonitrile, the specific activity of the remaining toluene had been lowered by 1.9 and 2.7 % at the yields 32 and 41 %, respectively. This change is within the experimental error and corresponds to $\beta = 1.17$ and $\beta = 1.18$ if $q = 1.45$, this value corresponding to 58 % ortho isomer. In view of the experimental error it could be concluded that $\beta = 1.2 \pm 0.2$. Since toluene is less reactive than anisole, there is considerably less reason to fear that insufficient mixing could have influenced these results.

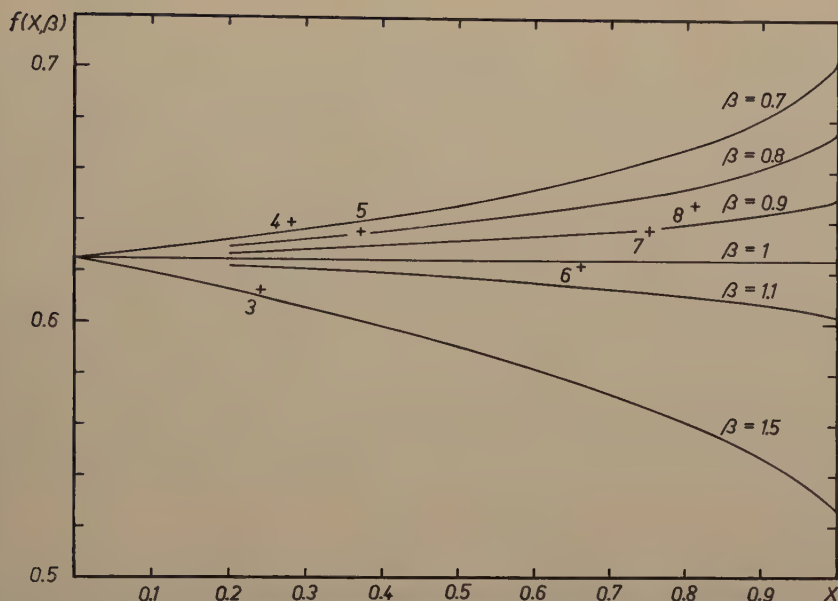


Fig. 1. Tritium content of the mixture of nitroanisoles relative to that of initial anisole-2-*t*. The curves show the function $f(x, \beta)$ for certain values of β . (The numbers of the experiments are the same as in Table 1.)

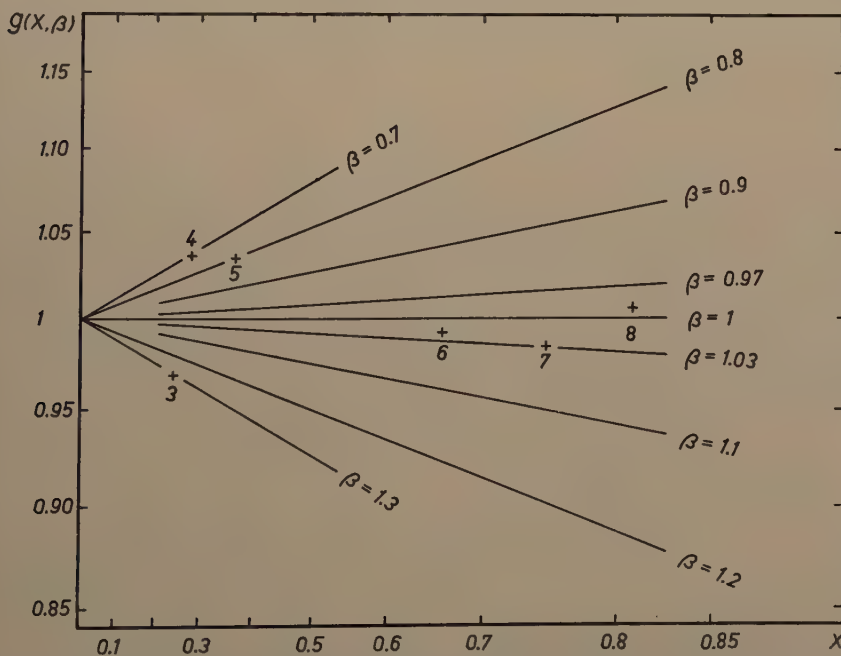


Fig. 2. Relative change in tritium content of anisole-2-*t* in the reaction (double-logarithmic representation). The straight lines show the function $g(x, \beta)$ for certain values of β . (The numbers of the experiments are the same as in Table 1.)

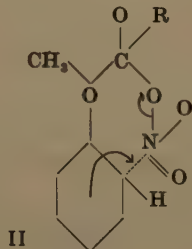
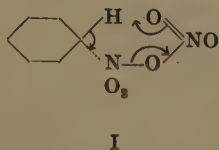
Discussion

The results on anisole corroborate the work of Griffiths, Walkey, and Watson [1] and that of Burton and Praill [2]. Under the conditions recommended by the latter the percentage of ortho isomer becomes as high as 75 %. The amount of meta isomer was found to be less than 0.5 %, and it is probably of a considerably smaller order of magnitude in view of the results obtained in bromination [13] and hydrogen exchange [14]. In nitrations with nitric acid in various media, Bunton, Minkoff, and Reed found the same proportion to be inferior to 0.1 % [15].

With reagents generally known to give rise to nitronium ions, considerably lower amounts of *o*-nitroanisole are formed [1, 15]. The temperature has generally been higher than in the present experiments, and the ortho proportion could be supposed to rise with temperature when this proportion is less than the statistical value (two thirds when meta can be neglected). A figure comparable to the 75 % with benzoyl nitrate seems to be about 30 %. The proportions in the nitration of toluene have no such dependence on the nitration conditions; the results of Table 2 do not deviate significantly from those under ordinary conditions. This agrees with earlier observations [16].

Nitration through nitrosation, which occurs with substances like anisole and phenol [5], tends to give high yields of the para derivative. It is difficult to know to which extent this effect has depressed the amount of ortho isomer in the "nitronium ion" experiments. With phenol, the percentage of the ortho isomer varies between 9 and 73 when the ratio between nitric and nitrous acid changes from 0.5 to infinity in sulphuric acid solution [5]. The variation in the isomeric proportions of nitroanisole covers an interval less than that for phenol. It is thus impossible to draw any definite conclusions about the reason for the variation in the orientation in anisole.

The main purpose of the present investigation was to measure the hydrogen isotope effect in the nitration of anisole which gives a high yield of the ortho derivative. According to current theories, the acyl nitrates nitrate by themselves [2] or through dinitrogen pentoxide [3]. For the reaction between the latter and the aromatic nucleus, a special kind of concerted mechanism (I) has been proposed [9]. Such a mechanism would inevitably give rise to a kinetic isotope effect, because the carbon-hydrogen bond is opened in the rate-determining step. Consequently it is ruled out by the present result $k_T/k_H = 1.0 \pm 0.1$ at 0°C. Of course the result does not rule out dinitrogen pentoxide as the attacking entity, it shows only that the hydrogen replacement proceeds as in ordinary nitronium ion nitrations, i.e. the addition to the carbon is slow and the splitting off of the hydrogen is kinetically insignificant [4, 6].



In view of the results with pure nitronium ion nitration of phenol [5] one might perhaps consider 75 % as a "normal" proportion of ortho isomer and ascribe the lower percentages with nitric acid to a nitrosation mechanism, which does not occur with toluene. The reason for this difference is primarily that nitrous acid could be produced in nitric acid oxidation of anisole, while an analogous reaction does not take place with toluene.

An at least superficially attractive explanation of the different orientations in anisole would also be the possibility of complex formation between the reagents, dinitrogen pentoxide or benzoyl nitrate, with anisole due to its ether oxygen atom, which has no equivalent in toluene. In order to promote substitution in the ortho position, such a complex formation would have to stabilize the transition state relative to the initial state for the ortho position or at least to do so to a higher extent for the ortho than for the para position. Such a model for the formation of the transition state in the ortho position is shown (II). The arrow originating in the ring indicates localization of two of the π -electrons at the ortho carbon atom to form an sp^3 hybridization. The hydrogen atom at the same carbon is assumed to remain in its bond (finally sp^3) throughout the slow addition step. It is to be observed that the created six-membered ring¹ cannot be a coplanar "aromatic" ring, since the carbon and nitrogen have four ligands. The model applies equally well to dinitrogen pentoxide, if the C and R of the benzoyl nitrate are replaced by nitrogen and oxygen, respectively. Remaining attached to the methoxy group, the residue of the nitrating molecule might subsequently and rapidly accept the hydrogen by a similar ring mechanism and form benzoic or nitric acid, respectively. It must be admitted, however, that it is difficult to see why the creation of the bond with the methoxy oxygen atom should promote the reaction. The electronegativity of the oxygen will be increased and that of the carbon (or corresponding nitrogen) decreased. Such a change will hardly promote the reaction, especially as no resonance energy is gained in closing the ring.

Summary

The isomeric proportions in the nitration of anisole with nitric acid in acetic anhydride, with benzoyl nitrate in carbon tetrachloride, and with benzoyl chloride and silver nitrate in acetonitrile have been determined by infra-red spectrophotometry. Earlier results are corroborated, and the maximum percentage of *o*-nitroanisole in the product was 75, obtained under the last-mentioned conditions. Under the same conditions it was shown that no isotope effect arises; $k_T/k_H = 1.0 \pm 0.1$ at 0°C.

The orientation in toluene does not show the same change with conditions as that in anisole, and no isotope effect occurs.

The implications of the results are discussed in the light of current theories.

The Swedish Natural Science Research Council has contributed to the acquirement of the infra-red spectrographic equipment, which is gratefully acknowledged.

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¹ The possible importance of the six-membered ring in this connection was proposed by Docent Arne Brändström in an interesting discussion.

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Chromatography of deoxyribonucleic acid on calcium phosphate

By GIORGIO SEMENZA¹

With 3 figures in the text

DNA chromatography offers special problems, similar to those encountered in protein chromatography. Their high molecular weight prevents them from entering the finest pores of the adsorbing materials, they are easily denatured (possibly by the distortion due to the steric configuration of the active sites of the adsorbing materials), and their poor stability prevents the use of excessive pH values or of many solvents. Moreover, the desorption is often difficult or impossible. In the case of DNA,² an additional difficulty resides in the strong intermolecular interactions.

It is not surprising therefore that only very few methods of column chromatography of DNA have been reported. Most of these are based upon the DNA-protein affinity, and histone (1, 2) or methylated globine (3) columns have been used. So far only one successful attempt has been reported on a non-protein material, the ECTEOLA-cellulose (4, 5).

In the present study, another adsorbing non-protein material was used, a calcium phosphate (brushit-hydroxyapatite) gel, that has been successfully employed for protein chromatography (6).

Analytical and preparative methods

Total N was determined according to a modification of Kjeldahl's method (7), total P according to Schaffer's method (8) and protein content according to Lowry's modification of Folin reaction (9).

Purine and pyrimidine composition of DNA was estimated, after acid hydrolysis (Wyatt, 10), by paper electrophoresis (11).

Calf thymus DNA was prepared according to Kay *et al.* (12); it had a N/P ratio of 1.6.

Chromatographic procedure

Calcium phosphate was prepared according to Tiselius *et al.* (6); in order to have a high flow rate, the smallest particles were discarded, and no pressure was used to

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² Abbreviations used: DNA: deoxyribonucleic acid. DNP: Deoxyribonucleoprotein. EDTA: ethylen-diamine-tetra-acetate, Na salt.

pack the columns. All experiments were carried out at high ionic strength (1 *M* NaCl), in order to reduce DNA's intermolecular interactions. The presence of NaCl does not interfere seriously in the adsorption and elution process on calcium phosphate.

Samples of DNA were dissolved in 0.05 *M* potassium phosphate buffer, containing 1 *M* NaCl, pH 6.3, centrifuged, and the supernatant applied to a column of calcium phosphate, which had been equilibrated overnight against the same buffer.

In order to have the best resolution, very small amounts of DNA were applied to comparatively large columns, although the working capacity of calcium phosphate for DNA is of the same order of magnitude as for the proteins (about 5 mg/ml Ca phosphate gel).

Since stepwise elution was found to be more effective than continuous gradient elution, the former was used in almost all the experiments. As eluents, solutions of increasing concentrations of phosphate buffer were used; the molarity of NaCl (1 *M*), and the pH (6.3) were kept constant. The last washing was performed with 3 *M* phosphate buffer, containing 0.007 *M* EDTA. All experiments were carried out at room temperature.

Results

Chromatography of DNA (Fig. 1)

0.25 mg of calf thymus DNA dissolved in 2 ml of 0.05 *M* potassium phosphate buffer (+ 1 *M* NaCl, pH 6.3), was applied to a column (1.6 × 5 cm), and the elution was carried out as described above. The flow rate was about 0.7 ml/min., and fractions were collected every 5 min. The results are given in Fig. 1*a*, as the extinction at 260 *mμ* plotted against the tube number. The arrows and the numbers indicate the concentrations and the stage of elution where the eluents were applied to the column.

As it is seen from the figure, two peaks were obtained (a third peak, not shown in the figure, was obtained with the last washing with phosphate + EDTA). "0.30 *M*" and "0.35 *M*" fractions contained each approximately 40% of the original DNA, and another 5% was eluted with the last phosphate-EDTA washing.

After dialysis overnight against the starting buffer, the two major peaks were rechromatographed (under identical conditions with the same batch of Ca phosphate), with almost quantitative recovery. The rechromatography of the "0.35 *M*" fraction is shown in Fig. 1*b*, and that of "0.30 *M*" fraction in Fig. 1*c*. The latter was further rechromatographed and the results are given in Fig. 1*d*.

As can be seen, the "0.35 *M*" fraction behaves as a stable and reproducible fraction, whilst the "0.30 *M*" fraction also gives rise to a considerable amount of the "0.35 *M*" fraction even on repeated chromatography.

Some of the "0.35 *M*" fraction were isolated and compared in its ultracentrifugal behaviour with the original DNA, using 0.326% solutions (in 0.35 *M* potassium phosphate buffer, + 1 *M* NaCl, pH 6.3). Under these conditions, sedimentation constants of 1 and of 5.6 were obtained for the original DNA and the "0.35 *M*" fraction, respectively.

The third fraction (eluted by phosphate + EDTA) behaved as a reproducible component in rechromatographic experiments.

No difference in the purine and pyrimidine composition could be shown between the original DNA material and the fractions (see table).

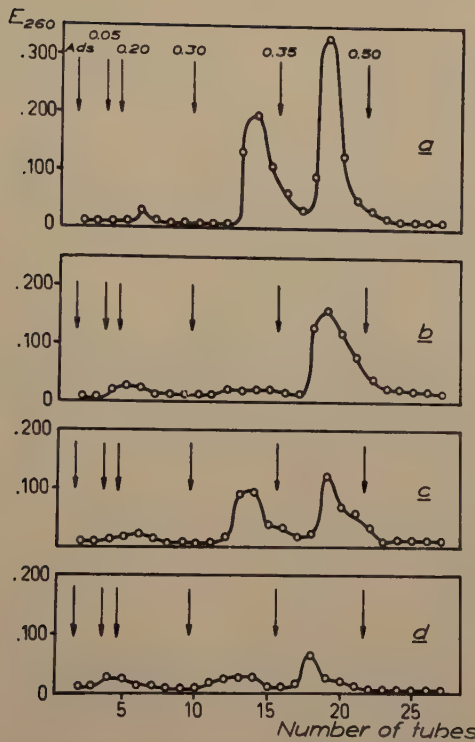


Fig. 1. Chromatography of calf thymus DNA on Ca phosphate. The columns (1.6×5 cm) were equilibrated against 0.05 *M* K phosphate buffer + 1 *M* NaCl, pH 6.3; their dead volume corresponded to about three fractions. As eluting agents, potassium phosphate buffers, all containing 1 *M* NaCl, pH 6.3, were used; their molarities and the stage at which they were applied to the top of the columns are indicated by the numbers and the arrows in the upper part of the figure. (a) Chromatography of 0.25 mg DNA. The sample was applied dissolved in 2 ml 0.05 *M* K phosphate buffer + 1 *M* NaCl, pH 6.3 (first arrow). (b) Rechromatography of the fraction eluted by 0.35 *M* phosphate buffer (+ 1 *M* NaCl), in experiment reported under *a*. (c) Rechromatography of the fraction eluted by 0.30 *M* phosphate buffer (+ 1 *M* NaCl), in experiment reported under *a*. (d) Re-rechromatography of the fraction eluted by 0.30 *M* phosphate buffer (+ 1 *M* NaCl), in experiment reported under *c*. For other details, see text.

Table 1. Purine and pyrimidine composition of the fractions obtained from calf thymus DNA by chromatography on calcium phosphate.

The values are expressed as per cent of total moles recovered. The last figure is uncertain. Abbreviations: A, adenine; T, thymine; G, guanine; C, cytosine; 5MC, 5-methyl-cytosine.

	A	T	G	C + 5MC	$\frac{A+T}{G+C+5MC}$
Original calf thymus DNA	29.0	28.5	21.2	21.3	1.35
Fractions eluted with 0.30–0.35 <i>M</i> K phosph. buffer + 1 <i>M</i> NaCl, pH 6.3 .	28.6	29.0	21.2	21.2	1.36
Fraction eluted with 3 <i>M</i> K phosph. buffer + 0.007 <i>M</i> EDTA	29.2	28.8	21.2	20.8	1.34

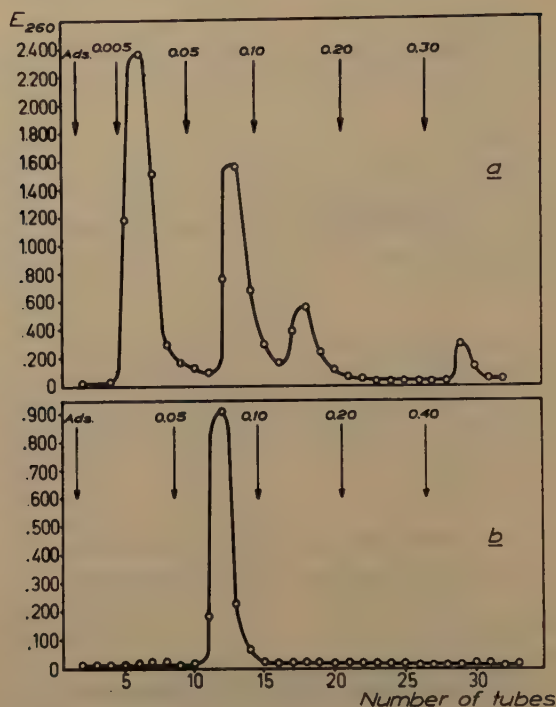


Fig. 2. Chromatography of a partial hydrolysate of DNA on Ca phosphate.

(a) 3 mg of DNA (hydrolysed in 1 ml 0.1 *N* HCl at 100°C for 15 min.) in 5 ml 0.005 *M* K phosphate buffer + 1 *M* NaCl, pH 6.3, were applied to a column (1.6 × 5 cm) of Ca phosphate (first arrow). For other explanations, see under Fig. 1. (b) Rechromatography of a part of the fraction eluted by 0.05 *M* K phosphate buffer (+ 1 *M* NaCl), in the experiment reported under *a*.

Fractionation of a partial hydrolysate of DNA (Fig. 2)

3 mg of calf thymus DNA were hydrolysed in 1 ml 0.1 *N* HCl at 100°C for 15 min. After neutralisation, the hydrolysate was brought to 5 ml with 0.005 *M* potassium phosphate buffer + 1 *M* NaCl (pH 6.3) and chromatographed on a column of calcium phosphate (1.6 × 5 cm). Fractions of 3 ml were collected every 5 min.

Of the four peaks obtained, one was not adsorbed (tubes 5–10), whereas the three others were eluted by 0.05 *M* (tubes 11–16), 0.10 *M* (tubes 17–19) and 0.30 *M* (tubes 29–30) potassium phosphate buffer (+ 1 *M* NaCl, pH 6.3), respectively. The over-all recovery was about 95 %.

Part of the fraction eluted with 0.05 *M* buffer was diluted 1:10 with NaCl, and rechromatographed as above (see Fig. 2*b*). As can be seen, a real reproducible fractionation was achieved.

Each of the fractions from the experiment reported in Fig. 2*a* was dialysed overnight against 0.005 *M* potassium phosphate buffer (+ 1 *M* NaCl, pH 6.3). It was shown that the non-adsorbed fraction (presumably largely composed of free purines) is entirely dialysable; the fraction eluted by 0.05 *M* buffer is composed of dialysable and non dialysable fragments; the other two fractions are almost completely non-dialysable.

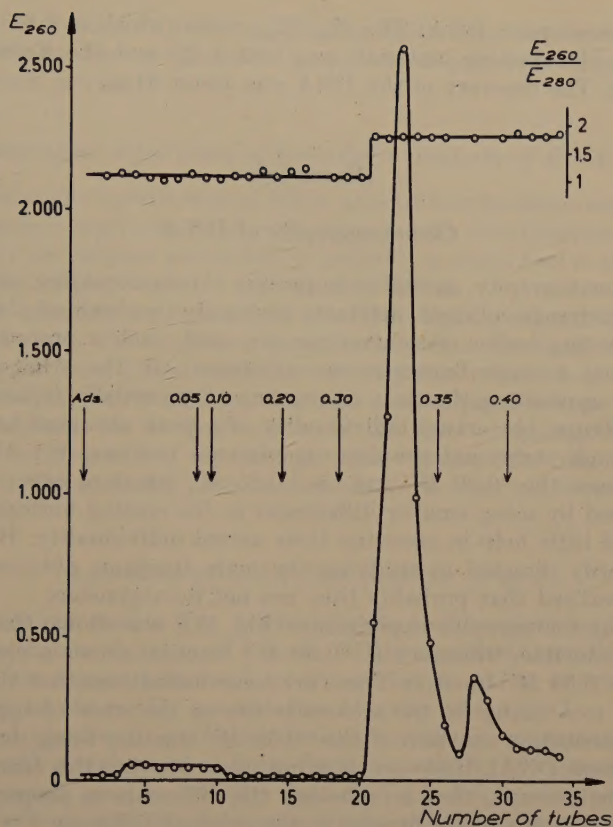


Fig. 3. Chromatography of a crude DNP preparation on Ca phosphate. Crude DNP from 1 g of calf thymus was chromatographed on a column of 1.6×5 cm. In the upper part of the figure, the E_{260}/E_{280} ratio is reported; it was 1.74 in the starting material, and it is 1.84 in pure DNA.

Chromatography of a crude DNP preparation (Fig. 3)

1 g of frozen calf thymus was homogenized in 0.14 *M* NaCl + 0.01 *M* Na citrate (200 ml) and centrifuged in the cold. The sediment was washed with 200 ml of the same solution, suspended in 30 ml of 1 *M* NaCl + 0.05 *M* potassium phosphate buffer, pH 6.3, and extracted overnight at $+4^{\circ}\text{C}$.

After centrifugation, the supernatant (which had an ultraviolet extinction ratio of $E_{260}/E_{280} = 1.74$) was applied to a column of calcium phosphate (1.6×5 cm) and chromatographed as above. During the adsorption, a strong decrease in the rate of flow occurred; hence, enough pressure was applied to produce a rate of flow of about 10 ml per hour. Fractions were collected every 30 min.

As can be seen in Fig. 3, at least two fractions were obtained: one that was not adsorbed (tubes 4–10), and another which was eluted by 0.30–0.35 *M* potassium phosphate buffer (+ 1 *M* NaCl, pH 6.3) (tubes 21–34). The first fraction contained almost all the protein of the original sample, whereas the second peak was largely

composed of almost pure DNA. The E_{260}/E_{280} ratio (which is 1.84 for pure DNA, and was 1.74 in the starting material) was 1.82–1.83, and the Folin test was only slightly positive. The recovery of the DNA was about 94 %.

Discussion

Chromatography of DNA

In DNA chromatography, as well as in protein chromatography, on calcium phosphate or ionic exchange columns, artefacts can easily be obtained, if too small differences in the eluting buffer concentrations are used: such a procedure would give many peaks from a single homogeneous substance. On the other hand, different substances may appear together in a single zone only partially separated by mutual displacement. Hence, the actual individuality of a peak obtained has to be judged essentially through rechromatographic experiments (see also (6)). Also, though it is likely that between the "0.30 *M*" and the "0.35 *M*" fractions other "mixed" peaks could be obtained by using smaller differences in the eluting buffer concentrations, this would be of little help in asserting their actual individuality. Hence, attention has been primarily directed in studying the main fractions obtained, as a whole; although it is realized that probably they are not homogeneous.

From the rechromatographic experiments (Fig. 1) it was shown that the "0.35 *M*" fraction is reproducible, whereas the "0.30 *M*" fraction shows a clear tendency to give rise to the "0.35 *M*" fraction. There are some indications that this phenomenon may be related to a change in the molecular size or the state of aggregation of the DNA (the sedimentation constant of the "0.35 *M*" fraction being much higher than that of the original DNA). However, it is not clear whether this transformation is a spontaneous phenomenon, that is, whether the difference in properties responsible for the fractionation is already present in the original DNA, or if it is due to some factor acting during the chromatographic procedure. In this connection, it may be recalled, for example, that Ca ions are known to have a "stabilizing" effect on DNA. Some experiments were carried out to investigate this problem, but no conclusive evidence could be reached.

The third fraction (eluted by 3 *M* potassium phosphate + 0.007 *M* EDTA, and representing less than 5 % of the total DNA; not reported in the figure) was shown to be reproducible by rechromatography.

The purine and pyrimidine composition of the "EDTA fraction", of the "0.30 *M*–0.35 *M*" fractions, and of the original DNA, is the same (see Table 1). In this respect, therefore, no fractionation has been obtained among the DNA species differing in purine and pyrimidine composition (cf. instead (1, 2)). This is not surprising, since the binding of the DNA with cations is known to be predominantly a function of the phosphoryl residues, and a difference in purine and pyrimidine composition may be expected to have little, if any, effect on it.

Chromatography of DNA on Ca phosphate has remarkable similarities with protein chromatography on the same material. Thus small changes in the concentration of the eluant bring about large changes in R_f , so that elution takes place at very high R_f values. This behaviour corresponds to what can be foreseen when many small ions compete for the active sites of the adsorbing material with one polyelectrolyte ion (6). In this case, in the exchange equilibrium equation of the adsorption–elution

process, the concentration of the small eluting buffer ions enters at a power which increases with the number of the active groups in the polyelectrolyte involved in the adsorption-elution process.

Chromatographic separation of breakdown products of DNA (Fig. 2)

From a partial acidic hydrolysate of DNA, at least four fractions could be obtained. Rechromatographic experiments showed that a real fractionation was achieved. Chromatography on calcium phosphate is probably a useful tool in the fractionation of high molecular weight breakdown products of DNA.

From dialysis experiments it could be seen that, besides other differences (in composition, for instance), the fragments separated by this procedure mainly differ as to their molecular size. These data, as well as those obtained on the sedimentation behaviour of the "0.35 *M*" fraction (see above) provide some indication that on calcium phosphate the molecular size or the state of aggregation of the DNA molecules may be responsible, at least in part, for the fractionation obtained. It may be recalled that a similar phenomenon has been reported for some proteins by Tiselius *et al.* (6).

Separation of DNA from proteins (Fig. 3)

The experiment reported in Fig. 3 demonstrates that an almost protein-free DNA was obtained by chromatography of a crude DNP preparation on calcium phosphate. This procedure has earlier been used by the author to purify enzyme preparations from RNA, with an essentially quantitative recovery of both (13).

Only a limited number of gentle methods are available to separate nucleic acids from proteins, with a high yield of both. Since the affinity of calcium phosphate for the proteins is much smaller (cf. (6)) than that for DNA, adsorption on calcium phosphate can be suggested for such separations, either by column chromatography when a small amount of material is available or by batchwise operation for larger amounts.

Summary

Deoxyribonucleic acid and its partial hydrolysis products have been chromatographed on calcium phosphate (hydroxyapatite) columns. The method is discussed and proposed, also, for the mild separation of DNA from proteins.

Note added in proof.—When this paper was already sent in for publication, I was informed that another technique for chromatography of DNA on Ca phosphate had been reported by Main and Cole (129th Meeting of the American Chemical Soc., Dallas, Texas, April 1956). From the information available, however, it appears that their method differs in more than one respect from that reported here, both in procedure and in the results obtained.

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